이학박사학위논문

군소 뉴런 배양을 이용한 CPEB4 가 관여하는 기억 경화와 단백질 분해가 관여하는 재경화 과정에 대한 연구

Studies on CPEB4-mediated memory consolidation and protein degradation-mediated reconsolidation using *Aplysia* neuronal cultures

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ABSTRACT

Studies on CPEB4-mediated memory consolidation and protein degradation-mediated reconsolidation using *Aplysia* neuronal cultures

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Dynamic process of memory requires consolidation to store memory in long-term. The memory reconsolidation hypothesis suggests that this memory trace becomes labile after retrieval and needs to be reconsolidated before it can be stabilized. However, it is unclear from earlier studies whether the same synapses involved in encoding the memory trace are those that are destabilized and restabilized after the synaptic reactivation that accompanies memory retrieval, or whether new and different synapses are recruited. To address this issue, I focused on simple form of non-associative memory, long-term sensitization of the gill- and siphon-withdrawal reflex in *Aplysia*. Using its cellular analog, long-term facilitation (LTF) at the sensory-to-motor neuron synapse, I found that on the cellular level, long-term facilitation at the sensory-to-motor neuron synapse that mediates long-term sensitization is also destabilized by protein degradation and is

restabilized by protein synthesis after synaptic reactivation, a procedure that parallels memory retrieval or retraining evident on the behavioral level.

In addition, I also focused on the issues that two pharmacologically distinct types of local protein synthesis are required for synapse-specific LTF in *Aplysia*: one for initiation and the other for maintenance. ApCPEB, a rapamycin sensitive prion-like molecule regulates a form of local protein synthesis that is specifically required for the maintenance of the LTF. However, the molecular component of the local protein synthesis that is required for the initiation of LTF and sensitive to emetine is not known. Here, I identified a homolog of ApCPEB responsible for the initiation of LTF. The ApCPEB homolog which we have named ApCPEB4-like protein is responsive to 5-hydroxytryptamine (5-HT), lacks a prion-like domain, and is translated (but not transcribed) in an emetine-sensitive but rapamycin-insensitive and PKA-dependent manner. The ApCPEB4 binds to different target RNAs than does ApCPEB. Knock-down of ApCPEB4 blocked the induction of LTF, whereas overexpression of ApCPEB4 reduces the threshold of the formation of LTF. Thus, our findings suggest that the two different forms of CPEBs play distinct roles in LTF; ApCPEB is required for maintenance, whereas the ApCPEB4, which lacks a prion-like domain, is required for initiation.

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Keywords : CPEB, LTF, Local protein synthesis, Retrograde signaling, EPSP

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CHAPTER I

INTRODUCTION

BACKGROUND

Aplysia as a model system to study implicit memory

Memory is one of the most important functions of the brain for animal to survive in environment. Memory is not just remembering the food that I had for breakfast but it also defines who we are. Diseases related with memory such as Alzheimer's disease is thought as a serious because it makes us loose our self.

Because of its importance, a number of phycologist and biologist have been focused on understanding the memory formation and storage for the last 50 years. There are two major memory system in the brain. One is explicit (or declarative) and the other one is implicit memory (or procedural). Declarative memory is composed of episodic memory and semantic memory. The former is a conscious recall of the specific experience and the latter is that of the fact. And This Declarative memory requires brain region call hippocampus (Milner et al., 1998; Squire, 2004). On the other hand, implicit memory is learned motor skills or simple reflex such as learning to ride bicycle. This type of memory even exists in invertebrate.

By Ramon y Cajal's postulate(López-Muñoz et al., 2006) that learning result from changes in the strength of the synapse, neuroscientist started to focus on finding the mechanism of memory formation and storage in a simple memory system, implicit memory using simple model system such simple reflex forms of reflex learning in invertebrates including gill-withdrawal reflex of *Aplysia.* (Castellucci et al., 1970). A light touch to the siphon with tactile stimulus such as

brushing cause the siphon to contract and gill to withdrawal. Sensitization of the gill-withdrawal reflex is observed by applying noxious stimulus such as electric shock to the tail which makes stronger withdrawal reflex of both siphon and gill. Space repetitive training make short-term memory which last for min into long-term sensitization memory last for up to several weeks in aplysia (Frost et al., 1985; Greenberg et al., 1987).

Aplysia was one of the most suitable model systems because it was relatively simple to search the changes in the synaptic site of the circuit that mediate reflex after memory storage (Kandel and Spencer, 1968; Kandel, 2012). It offers three important advantages; First, neural circuit is simple. The number of nerve cell is only 20,000 whereas mammalian brain has a trillion nerve cells. And size of cells is big up to 1mm which is even visible with naked eye. Because of the size and morphology, major cells and its circuit involved in the reflex behavior is easily identifiable (Frost et al., 1985; Mackey et al., 1989; Kandel, 2001a). And it is also possible to reconstitute the system in a culture dish by making sensory-to-motor neuron monosynaptic coculture and study cellular basis of behavior change by learning. Second, electrophysiological recording of *Aplysia* neuronal cell culture is easy. Experimenter can record the neuron for long time because of the small tip size which makes only small damage to the cell and even multiple times for several days to tract the synaptic changes. Third, it is very simple to microinject the small molecules such as DNA, antibody, inhibitor, dsRNA into the cell which make it easy to study molecular manipulation of certain genes or protein related with synaptic plasticity.

Memory reconsolidation

Short-term memory become Long-term memory by a process called consolidation which is a protein synthesis dependent(Flexner and Flexner, 1966; Davis and Squire, 1984; Bailey et al., 1992). It was widely believed that long-term memory is permanent after its consolidation (Nadel and Land, 2000). But recently it has been shown that consolidated memory undergoes again a new consolidation process whenever it is reactivated with retrieval signal to retain memory (Nader et al., 2000a). It has been focused that what is the differences between consolidation and reconsolidation using variety of species including mouse, chick, rat, gerbil, human, pond snail (Alberini, 2005). For example, Protein synthesis in the central amygdala was required for consolidation but no for reconsolidation (Bahar et al., 2004). C-fos level was elevated in different brain region during consolidation and reconsolidation (Tronel and Sara, 2002). BDNF was required for consolidation but reconsolidation and zif268 was required for reconsolidation but consolidation in hippocampus contextual fear conditioning (Lee et al., 2004). In contrast, it also shares some molecular mechanisms between two process. For example, CREB is required for consolidation and reconsolidation of contextual fear conditioning((Debiec and LeDoux, 2002; Kida et al., 2002). Hippocampal MAPK, Amygdala PKA and zif268 has been reported to be required for both consolidation and reconsolidation (Bozon et al., 2003; Kelly et al., 2003; Koh and Bernstein, 2003).

How memory become labile after reactivation? While researches in reconsolidation field focused on the “re-consolidation” process, it was not well studied how labile state is reinstated. Recently, it has been shown that consolidated

memory become labile after memory retrieval by ubiquitin proteasome dependent synaptic protein degradation in mouse hippocampus CA1 (Lee et al., 2008a; Kaang et al., 2009). However, it was not possible to address whether this destabilization and restabilization of memory occur in the same synapse that stored memory in this complex mammalian system. In this thesis I address this question at the cellular level using a simple model system called *Aplysia* monosynaptic coculture.

Molecular mechanisms of long-term sensitization memory in *Aplysia*

How short-term memory become long-term memory? Whereas one training trial of electric shock to the tail induce a short-term memory lasting minutes, spaced repetitive training to the tail induce long-term memory lasting days to weeks (Carew et al., 1972; Pinsker et al., 1973). Using simple circuit of reflex circuit and monosynaptic cultures made with sensory and motor neuron in the reflex, it was possible to study the cellular and molecular mechanisms of short-term and long-term memory for sensitization (Castellucci et al., 1970; Montarolo et al., 1986; Cohen et al., 1997). This monosynaptic Glutamatergic coculture (Dale and Kandel, 1993; Trudeau and Castellucci, 1993) *in vitro* model system replicated the behavioral sensitization in a dish by replacing the tail shock with application of serotonin(5-HT) which is released by modulatory neuron in the intact animal circuit (Mackey et al., 1989; Marinesco and Carew, 2002). Single brief application of serotonin produce short-term increase of synaptic strength lasting minute, spaced repetitive application of serotonin produce long-term increase of synaptic strength lasting days to week (Montarolo et al., 1986). When the *Aplysia* tail is

activated by a shock, it activate modulatory neuron which release serotonin. This increase cAMP level and activate cAMP-dependent protein kinase A(PKA) by binding of cAMP to regulatory subunit in siphon sensory neuron. This catalytic subunit of PKA can phosphorylate potassium channel which increase the excitability and proteins involved in exocytosis which increase the release of neurotransmitter. When serotonin activation occur repeatedly, it increase the persistent level of serotonin even higher which then makes catalytic subunit of PKA to recruit MAPK. These proteins move to the nucleus and phosphorylate transcription factors and activate gene expression involved in the formation of long-term memory (Bacskai and Hochner, 1993; Martin et al., 1997c). PKA then activate ApCREB1 and express several immediate early genes which has pivotal role in induction of LTF such as Ubiquitin hydrolase (Hegde et al., 1997) and CCAAT-box-enhanced binding protein(C/EBP) (Alberini et al., 1994). These genes product then activate downstream gene expression for the synaptic growth with ApAF (Bartsch et al., 2000) which is constitutively expressed. While PKA activate ApCREB1 (Bacskai and Hochner, 1993), accompanying down regulation of ApCREB2 by MAPK is required for LTF formation (Martin et al., 1997c; Michael et al., 1998; Guan et al., 2003) (Fig. 1). In contrast to activity of kinase in formation of LTF, activity of synaptic protein phosphatase such as PP1 and calcineurin counterbalancing with PKA also play a key role in regulation of LTF balancing with kinases (Sharma et al., 2003).

How facilitation is maintained in a specific synapse? Since mRNA is transcribed in the nucleus by those immediate early gene, newly synthesized mRNA and protein involved in synaptic growth will diffuse to cell wide manner.

To address this question, “synaptic tag” model has been proposed(Frey and Morris, 1997; Martin et al., 1997a; Frey and Morris, 1998; Martin and Kosik, 2002; Redondo and Morris, 2011). In *Aplysia*, *Aplysia* homolog of CPEB, Local protein synthesis regulator, ApCPEB has been studied widely functioning as a tagging molecule at the activated synapse(Martin et al., 1997a; Casadio et al., 1999; Si et al., 2003a; 2003c; 2010a; Fiumara et al., 2015).

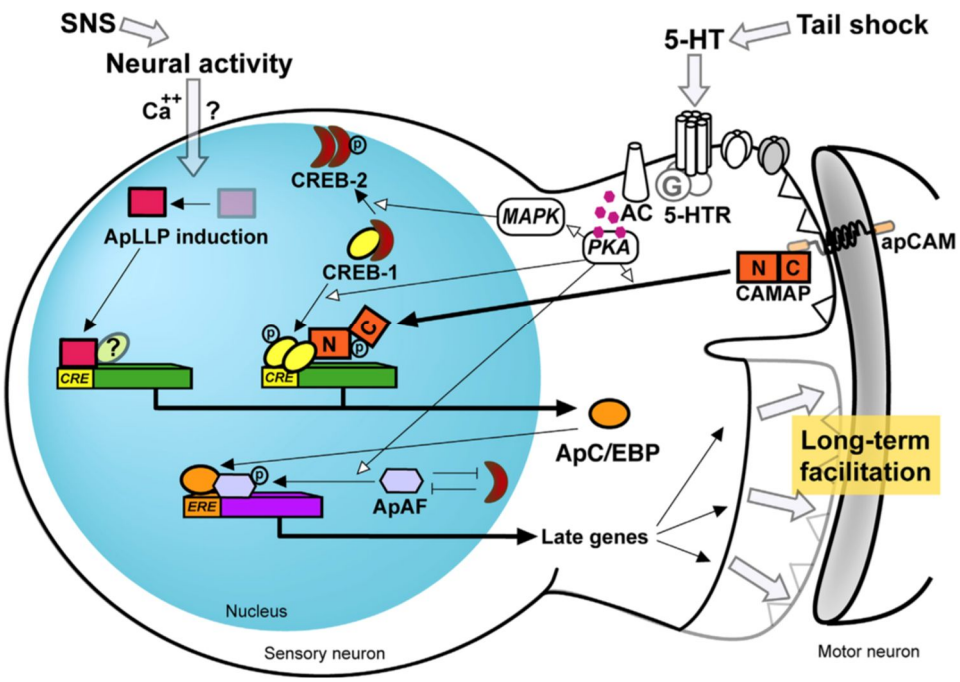


Figure 1. Molecular mechanisms of Long-term sensitization in *Aplysia*

Long-term sensitization memory is protein synthesis dependent. Activated PKA phosphorylate CREB-1 together with MAPK which phosphorylate CREB-2 in nucleus. Then, CREB-1 increase the expression of immediate early genes such as Ubiquitin hydrolase which turn PKA into persistent kinase and C/EBP which increase translation of late phase gene related with synaptic growth with ApAF. (adapted from Lee et al.,2008)

Functional role of CPEBs in learning and memory

Cytoplasmic polyadenylation is a key process that serves to unmask particular mRNAs and enables them to be translated. Cytoplasmic polyadenylation element binding (CPEB) protein bind to cytoplasmic polyadenylation element (CPE) in 3'-UTR of target mRNA and promote cytoplasmic polyadenylation. CPEB was first discovered in Xenopus oocyte. This CPEB increase translation of masked mRNA which is dormant transcripts in the oocyte during the completion of the meiotic divisions or in the early embryo. Vertebrate contain four members of the CPEB (CPEB1-4). Drosophila contain two CPEB family proteins (Orb and Orb2). C.elegans contain four CPEB family proteins (CPB1-3 and FOG-1). And two CPEB proteins in Aplysia has been reported (CPEB1 and 2). Vertebrate CPEB1 has high affinity to CPEs but CPEB2-4 does not. Studies using SELEX(selected evolution of ligands by exponential enrichment) revealed that CPEB3-4 bind to U-rich sequence (Huang et al., 2006a; Ivshina et al., 2014).

Because local protein synthesis is important in synaptic plasticity (Kang and Schuman, 1996), the functional role of CPEB proteins in learning and memory has been studies in many species. In *Mouse*, CPEB1 knockout mice study showed that LTP is impaired with theta burst stimulation protocol(Alarcon et al., 2004; Zearfoss et al., 2008). But interestingly did not show reduced learning ability. Instead, the mice exhibited impaired extinction (Berger-Sweeney et al., 2006). Several mRNAs such as CaMKII, tissue plasminogen activator(TPA), GluN2A in neurons are shown to undergoes activity dependent polyadenylation (Wu et al., 1998; Shin et al., 2004; DU and Richter, 2005). CPEB1 also has important role in transporting mRNA through microtubules with kinesin and dynein (Huang et al.,

2003). The other CPEB, CPEB3 is also important in learning and memory. CPEB3 total KO mice showed enhanced hippocampus dependent learning with increased level of PSD-95 and NMDAR (Chao et al., 2013). Other groups have found mono-ubiquitination of CPEB3 by neuralized1 affect reverse inhibitory effect of CPEB3 on GluA1 and GluA2 translation(Pavlopoulos et al., 2011). And other following studies using adult forebrain specific KO mice revealed that it shows impaired LTM and LTP. And prion-like mechanism of CPEB3 seems important for maintaining memory(Fioriti et al., 2015; Stephan et al., 2015). In contrast, CPEB4 KO mice did not show any observable effect on plasticity and learning and memory (Tsai et al., 2013).

In *Drosophila,* knockdown of Orb with RNAi impaired LTM in order-shock association paradigm (Pai et al., 2013). In addition, Orb2 deletion reduced male long-term courtship memory and poly-glutamine domain is important for this function (Keleman et al., 2007). In *Aplysia* CPEB, *Drosophila* Orb proteins and *mouse* CPEB3 contains Poly-glutamine domain. Especially in *Aplysia,* CPEB is important for maintenance of long-term facilitation and might form prion-like structure and constitute a tag at activated synapses (Si et al., 2003b; 2003d; 2010a; Raveendra et al., 2013)

In this thesis I also studied about new isoform of Aplysia CPEB which does not have prion-like poly-glutamine domain. I found that this new Aplysia CPEB, ApCPEB4 is important for initiation of LTF.

PURPOSE OF THIS STUDY

I was interested in molecular and cellular mechanisms of memory consolidation and reconsolidation. It has not been answered deeply how memory reconsolidation occurs at the synaptic level. In this thesis I address this question at the cellular level using aplysia monosynaptic coculture. In addition, I also studied about the functional role of new isoform of *Aplysia* CPEB in memory consolidation.

In chapter II, I focus on the cellular mechanism of memory reconsolidation. To investigate whether synaptic destabilization and restabilization occur in the same synapse where it stores memory, I firstly show the changes in synaptic strength after LTF inducing signal in *Aplysia* neuronal monosynaptic coculture system. Then, using pharmacology, I show the mechanism of synaptic destabilization and restabilization of synapse after retrieval signal in this system.

In chapter III, I focus on the role of ApCPEB4 for memory consolidation. I have cloned ApCPEB4 which is *Aplysia* homolog of CPEB4-like protein. I firstly show the expression of ApCPEB4 in central nervous system. Then, to investigate the role of show its distinctive role in long-term facilitation which is a cellular model of memory consolidation, Intracellular recording after knockdown or overexpression of ApCPEB4 in sensory neuron of *Aplysia* sensory-to-motor neuron coculture system was used. In addition, I show binding property of ApCPEB4 for the target mRNA and upstream molecule that activate ApCPEB4.

CHAPTER II

A cellular model of memory reconsolidation involves reactivation-induced destabilization and restabilization at the sensorimotor synapse in *Aplysia*

INTRODUCTION

The processes of memory reactivation (retrieval) have been the focus of several studies over the last decade. Retrieval is thought to return the memory to an unstable (labile) state, in which de novo protein synthesis-dependent reconsolidation is required to continue maintaining the memory over time (Nader et al., 2000b; Suzuki et al., 2004; Alberini, 2005; Dudai, 2006). Memory reconsolidation has been reported for a variety of memory paradigms in a number of different animal models (Nader et al., 2000b; Walker et al., 2003; Alberini, 2005; Lee et al., 2005); however, how memory reconsolidation works remains unclear.

At least two non-mutually exclusive hypotheses have been proposed (Alberini, 2011). One hypothesis suggests that reconsolidation is an updating process in which the synapses that encode the pre-existing memory are reorganized after memory retrieval in order to recruit new synaptic connections that allow the incorporation of new information (Dudai, 2004; Lee et al., 2008a; Kaang et al., 2009). The second hypothesis suggests a mechanism that is a continuation of the consolidation process at the same set of synaptic connections and that serves to strengthen memory, allowing it to become longer lasting and enduring and thereby preventing forgetting (Dudai and Eisenberg, 2004). Both of these views of reconsolidation are consistent with retraining or retrieval. In each case, synaptic reactivation could be implicit (e.g., during sleep) or explicit, and both would presumably have the same effect of making the memory stronger, more stable, and more resistant to post-retrieval interference.

Both types of reconsolidation hypotheses imply that the stored memory becomes labile after memory retrieval. To address how this occurs, we studied the retrieval of memories and found that they become labile via ubiquitin/proteasome-dependent synaptic protein degradation (Lee et al., 2008a; Kaang et al., 2009). Moreover, Doyère et al. (Doyère et al., 2007) found that inhibition of reconsolidation is correlated with reduced potentiation at reactivated synapses in the lateral amygdala. The foregoing studies suggest that signal retrieval activates protein degradation in the synaptic connections encoding the initial memory, and that protein synthesis is required for restoring or maintaining the memory. However, it remains unclear whether destabilization and restabilization after memory retrieval occur at the same synaptic connections where potentiation occurs for memory encoding (Lee et al., 2008a), or whether different synaptic connections are involved in the retrieval process (Sara, 2000; Dudai, 2004).

To address this issue, we used the elementary neural circuit that underlies sensitization of the gill- and siphon-withdrawal reflex, a simple form of nonassociative learned fear in Aplysia. A critical component of this reflex that contributes significantly to this behavior is the direct monosynaptic connection from the siphon sensory neuron to gill and siphon motor neurons. The sensory-to-motor neuron synapse can be reconstituted in dissociated cell culture, where it is modulated, as in the intact animal, by serotonin (5-HT), a modulatory transmitter released during sensitization training (Montarolo et al., 1986). Five applications of 5-HT over a period of 1.5 h—designed to simulate five shocks to the tail that produce long-term behavioral sensitization—produce both a long-term increase in the strength of the sensory-to-motor neuron synaptic connection lasting several

days which is called long-term facilitation (LTF) (Montarolo et al., 1986) and structural remodeling and growth of new sensory-to-motor neuron synapses (Glanzman et al., 1990; Bailey et al., 1992; Kim et al., 2003). The consolidation of both long-term sensitization and its cellular analog, LTF at the sensory-to-motor neuron synapse, requires de novo protein synthesis (Montarolo et al., 1986; Castellucci et al., 1989; Kandel, 2001a; Lee et al., 2008b; Kandel, 2012).

During sensitization training, stimulating the tail activates interneurons that release 5-HT onto the mechanoreceptor sensory neurons that innervate the siphon skin (Mackey et al., 1989), resulting in a strengthening of the sensory-to-motor neuron synapses that control the siphon-withdrawal reflex (SWR) (Wright et al., 1991; Sutton et al., 2001; 2002). Furthermore, the molecular mechanisms that govern behavioral long-term sensitization also govern the learning-related synaptic plasticity exhibited by the sensory-to-motor neuron synapses (Montarolo et al., 1986; Kaang et al., 1993; Kandel, 2001b; Lee et al., 2006). Therefore, the long-term memory (LTM) for sensitization in Aplysia and its sensory-to-motor neuron synapses are useful tools for studying fundamental properties of synapses, such as destabilization and restabilization after memory retrieval.

In this study, I investigated whether synaptic disruption and reconstruction are also necessary, at the same set of synaptic connections between the sensory and motor neurons that initially stored the memory, for maintaining LTF (the cellular analog of long-term memory of sensitization in siphon-withdrawal reflex) after synaptic reactivation protocols that mimic retrieval or retraining of the behavioral modification in the intact animal.

EXPERIMENTAL PROCEDURES

Cell Cultures

Sensory-to-motor cocultures and synapse recording followed previously described protocols (Montarolo et al., 1986; Lee et al., 2001; 2003). Sensory neurons isolated from the pleural ganglia of A. kurodai (100–150 g) were cocultured with LFS motor neurons obtained from the abdominal ganglia of adult animals (Montarolo et al., 1986).

Electrophysiology

At four days after sensory-to-motor coculture, the first EPSP measured by intracellular recording in an LFS motor cell by stimulating the sensory neurons with a brief depolarizing stimulus using an extracellular electrode (first recording). During EPSP measurement, the motor cell was impaled with a glass microelectrode filled with 2 M K-acetate, 0.5 M KCl, and 10 mM K-HEPES (10–15 MΩ), and the membrane potential was held at –40 mV be- low its resting value. Then the sensory-to-motor neuron synapses received five pulses of 5-HT (10 μM, 5 min/pulse, with a 15-min interval between pulses) to induce LTF. To reactivate the facilitated synapse, HA or five pulses of 5-HT treatment was performed. HA represents activation induced by four action potentials in the sensory neuron of the sensory-to-motor synapse with a 1-min interval. We used this multiple recording

paradigm to mimic stronger retrieval stimuli. To avoid providing long-term potentiation (Lin and Glanzman, 1994) or long-term depression (Lin and Glanzman, 1996) stimuli to sensory cells, a 1-min interval was applied. When the synaptic facilitation was examined at 120 h after the training (Fig. 7), half of the synapses were also tested at 24 h after the training to confirm the successful initiation of LTF; these synapses were no different from the other synapses at later testing time points (Fig. 8). To examine the effect of protein synthesis inhibition or proteasome inhibition after the reactivation, I applied emetine (100 μM; Sigma-Aldrich) or βlac (0.1 μM; Calbiochem) in the bath for 2.5 h immediately after the second recording. Bath application affects both the cell body and synapses simultaneously, which can serve to mimic our behavioral experimental conditions (i.p. injection) (Lee et al., 2012). To examine the effect of proteasome inhibition on basal synaptic transmission, I applied βlac (0.1 μM) or vehicle (0.1% DMSO) for 2.5 h immediately after the first recording, and performed the second recording 24 h later. When emetine or βlac was applied with 5-HT treatment, the inhibitor (emetine or βlac) was bath-applied at 30 min before the first application of 5-HT and remained in the bath throughout the 5-HT treatment and for 30 min after the 5-HT treatment. For the reactivation experiments, we excluded sensory-to-motor neuron synapses showing less than a 20% increase in synaptic strength on the second recording compared with the first recording.

Statistical Analyses

The effect of drug application across time points was examined using

two-way mixed (between- and within- subjects design) ANOVA with drug groups (between-subjects) and time points (within-subjects, repeated-measures) as factors.

Each drug application group was compared with other groups at specific recording time points using one-way ANOVA and the Newman–Keuls multiple-comparison post hoc test. In addition, one-sample t tests were used to compare the synaptic strength change with the basal level in each group.

RESULTS

Cellular model of memory reconsolidation in *Aplysia* monosynaptic culture

Based on our previous data showing reactivated fear memory becomes labile via ubiquitin/proteasome-dependent protein degradation(Lee et al., 2008a), I asked whether the same sensory-to-motor neuron synapse that was facilitated by five pulses of 5-HT also undergoes destabilization and restabilization after synaptic reactivation. To address this question, First, I decided to test new protein synthesis dependent synaptic restabilization occur after reactivation signal in our culture system.

I first measured the basal strength of the sensory-to-motor neuron synapse (first recording), and then induced LTF with five pulses of 5-HT. At 24 h after the 5-HT treatment, I retested synaptic strength (second recording) and found that it was significantly greater than the basal level (all groups, $P < 0.01$, one-sample t test compared with basal level of 0). After the second recording, we applied homosynaptic activation (HA) (Kandel, 2001a; Kim et al., 2003) as a reactivation (retrieval) signal by generating four action potentials (at 1-min intervals) in the sensory neuron of the sensory-to-motor neuron synapse. For the third recording (at 24 h after the second recording), the facilitated synaptic strength of the reactivated group (HA + vehicle group) was maintained and was significantly greater than the basal level ($P < 0.01$, one-sample t test compared with basal level), although the synaptic strength at the third recording showed a tendency toward a decrease

compared with the strength at the second recording (Fig. 2). However, combining HA with emetine after the second recording (HA + emetine group) disrupted the strength of the facilitated synapse, causing a reversion to its basal strength at the third recording ($P > 0.43$, one-sample t test compared with basal level) (Fig. 2). This result was consistent with our previous data showing new protein synthesis dependent reconsolidation after reactivation of long-term sensitization memory in *Aplysia* (Lee et al., 2012).

Vehicle application without HA (vehicle group) or emetine treatment without HA (emetine group) at the second recording did not disrupt the facilitated synaptic strength, and so the synaptic strength was significantly different from the basal level ($P < 0.05$, one-sample t test compared with basal level) (Fig. 3). The emetine only group, in which only emetine was applied without the second recording at 24 h after the first recording, also maintained synaptic strength at the third recording (Fig. 3). Although the group to which emetine was applied 24 h after the first recording exhibited some reduction in synaptic strength, this reduction was not statistically significantly different from that in either the vehicle or emetine only group. These results suggest that LTF at the sensory-to-motor neuron synapse reactivated by HA undergoes a reconsolidation phase after synaptic reactivation.

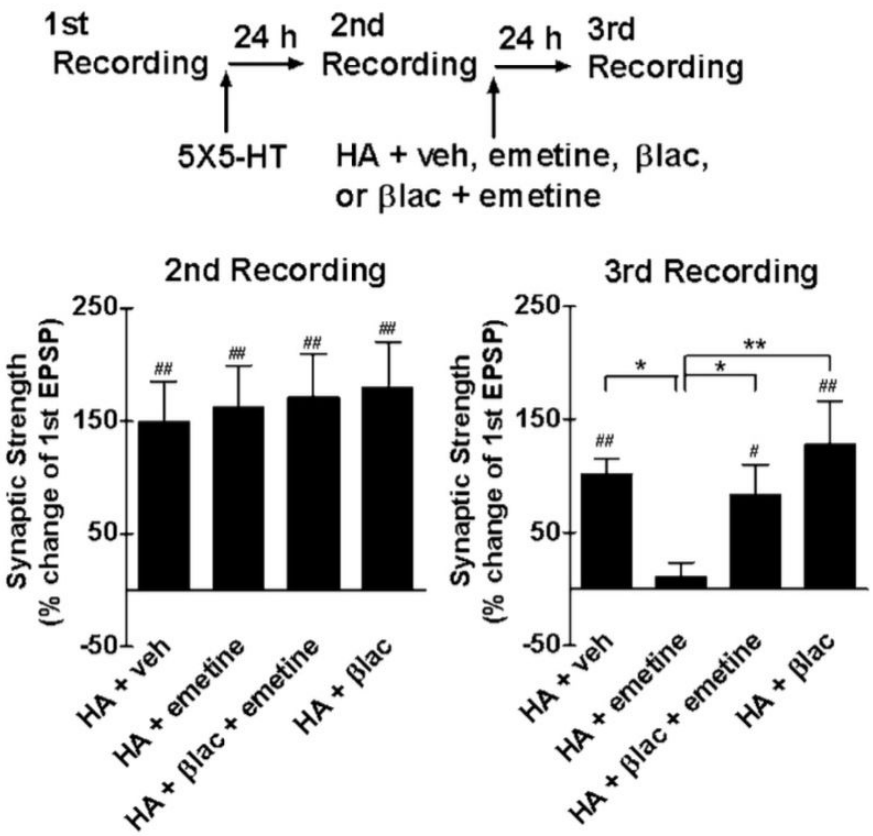

1st
Recording
24 h
2nd
Recording
24 h
3rd
Recording
5X5-HT
HA + veh, emetine, βlac,
or βlac + emetine
2nd Recording
3rd Recording
Synaptic Strength
(% change of 1st EPSP)
250
150
50
-50
##
#
*
**
HA + veh
HA + emetine
HA + βlac + emetine
HA + βlac

Figure 2. Synaptic destabilization and restabilization of LTF at the sensory-to-motor neuron synapse after reactivation by HA

(in collaboration with Sue-Hyun Lee)

(Upper) Schematic of the experimental procedure used for evaluating the effect of synaptic reactivation by HA on LTF.

(Lower) Bar graph showing mean percentage change ± SEM in EPSP amplitudes. (Lower, Left) On the second recording, the changes of EPSP amplitudes were not significantly different among groups. (Lower, Right) Compared with the vehicle application after HA (HA + veh; n = 8), emetine treatment after HA (HA + emetine; n = 10) impaired LTF on the third recording, whereas βlac treatment alone after HA (HA + βlac; n = 9) had no effect. However, βlac treatment immediately after the second recording prevented im- pairment of LTM induced by emetine treatment (HA + βlac + emetine; n = 8). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, one-sample t test compared with basal level. [$F_{(3,31)} = 4.38$, $P < 0.05$, one-way ANOVA], *P < 0.05, **P < 0.01, Newman–Keuls multiple-comparison test. There was no significant difference among groups in the EPSP amplitudes of the first recording [$F_{(3,31)} = 1.35$, $P > 0.27$; one-way ANOVA], and no significant correlation between the EPSP amplitudes of the first recording and the facilitated level of the second recording ($P > 0.11$, Pearson correlation).

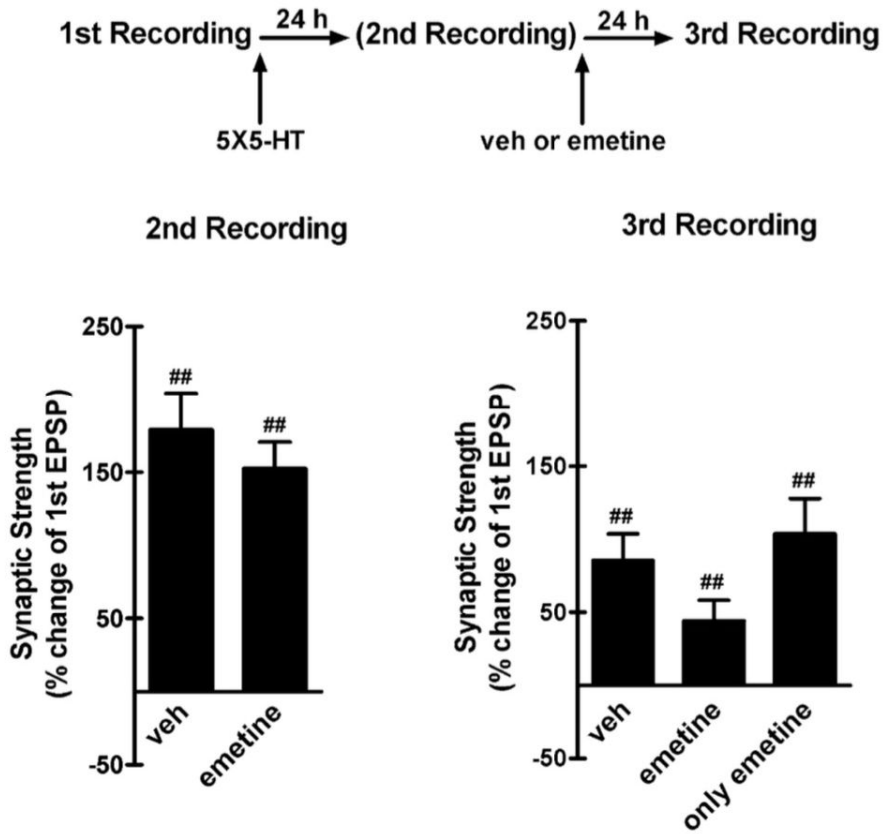

1st Recording
24 h
(2nd Recording)
24 h
3rd Recording
5X5-HT
veh or emetine
2nd Recording
3rd Recording
Synaptic Strength
(% change of 1st EPSP)
250
150
50
-50
##
##
veh
emetine
Synaptic Strength
(% change of 1st EPSP)
250
150
50
-50
##
##
##
veh
emetine
only emetine

Figure 3. Effect of emetine on maintaining synaptic strength without reactivation or retraining signal

Treatment with vehicle or protein synthesis inhibitor emetine without homosynaptic activation (HA) or 5 × 5-HT retreatment did not affect maintenance of facilitated synaptic strength for 48 h after the first recording.

(Upper) Schematic of the experimental procedure.

(Lower) Bar graphs showing mean percentage change ± SEM in excitatory postsynaptic potential (EPSP) amplitudes. On both the second and third recordings, changes in EPSP amplitudes were significantly different from the basal level in all groups ($^{\#\#}P < 0.01$, one-sample t test compared with basal level), and there was no difference among groups [$F_{(2,23)} = 3.16$, $P > 0.05$, one-way ANOVA]. veh, vehicle control group (n = 7); emetine, emetine control group (n = 13); only emetine, emetine-only treatment group without the second recording (n = 6).

Synaptic destabilization and restabilization occur in the synapse after reactivation stimulus in monosynaptic coculture

To determine whether ubiquitin/proteasome-dependent synaptic destabilization and protein synthesis-dependent synaptic restabilization occur at the same sensory-to-motor neuron synapse, we applied 0.1 μM βlac, which has no effect on basal synaptic strength or on consolidation of LTF, to the reactivated synapse using HA (Fig. 2 and Fig. 4). Concentration of βlac to use was tested before this experiment by comparing changes in basal synaptic strength between vehicle and βlac group (Fig. 5), because high concentration(10uM) of βlac can affect basal transmission(Zhao et al., 2003). Consistent with the behavioral results in *Aplysia* (Lee et al., 2012), βlac treatment prevented the disruption of LTF induced by emetine (HA + βlac + emetine), whereas βlac treatment alone (HA + βlac) had no effect (Fig. 2). One-way ANOVA with HA groups at the third recording revealed a significant effect of drug treatment, and application of the Newman–Keuls multiple-comparison post hoc test showed significantly lower synaptic strength in the HA + emetine group at the third recording compared with the HA + vehicle, HA + βlac + emetine, and HA + βlac groups (Fig. 2). Synaptic strength in the HA + βlac + emetine and HA + βlac groups was not significantly different from that in the HA + vehicle group at the third recording. Two-way ANOVA with time points (for the first and the third recordings) and drug groups as factors revealed significant effects of time points [$F_{(1,31)} = 40.96$, $P < 0.01$], drug groups[$F = 4.38$, $P < 0.05$], and the interaction between them[$F_{(3,31)} = 4.38$, $P <$

0.05]. These results suggest that consolidated LTF at the sensory-to-motor neuron synapse is destabilized via ubiquitin/proteasome- dependent protein degradation after synaptic reactivation (retrieval), and that synaptic destabilization and restabilization after synaptic reactivation occur at the same synaptic connections. Possible cellular signaling cascades such as alterations in intracellular Ca^{2+} levels, which may be recruited by HA of sensory neurons also might be involved in synapse destabilization.

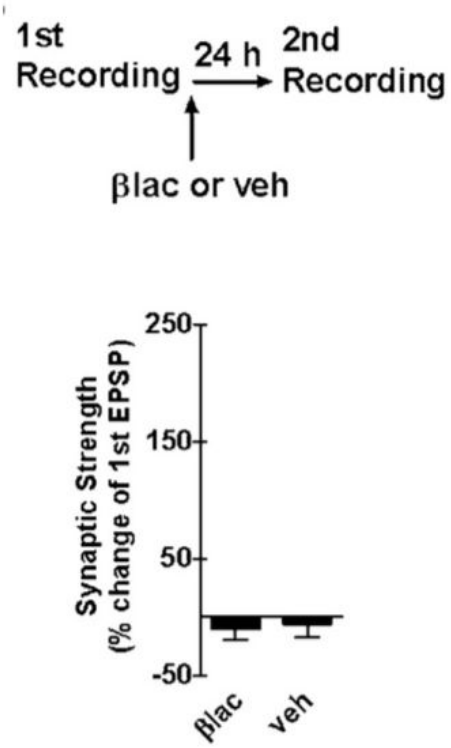


Figure 4. Effect of βlac on basal synaptic transmission

Proteasome inhibitor βlac did not affect basal synaptic transmission.

(Upper) Schematic of the experimental procedure used to evaluate the effect of βlac treatment on basal synaptic transmission.

(Lower) Bar graphs showing mean percentage change ± SEM in EPSP amplitudes. Neither βlac (n = 5) nor vehicle (veh; n = 6) treatment affected basal synaptic transmission.

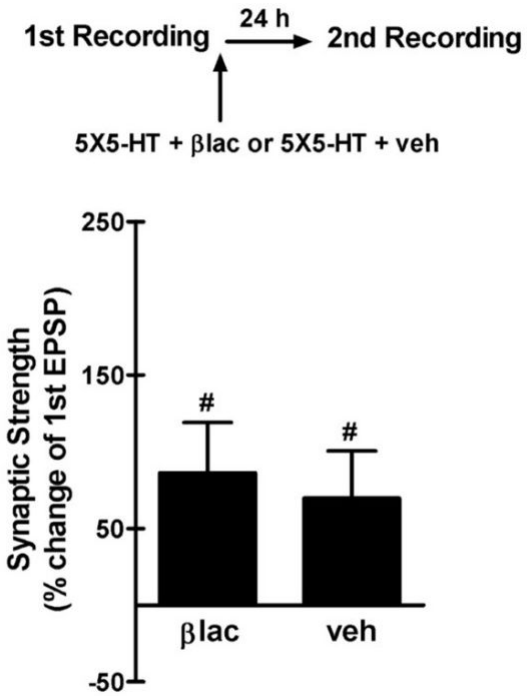


Figure 5. Effect of proteasome inhibitor, βlac on long-term facilitation

(in collaboration with Sue-Hyun Lee)

(Upper) Schematic of the experimental procedure used to evaluate the effect of βlac treatment on LTF consolidation induced by five pulses of 5-HT.

(Lower) Bar graph showing mean percentage change ± SEM in EPSP amplitude. The synaptic strength change was similar in the βlac treatment group (βlac; n = 16) and the vehicle treatment group (veh; n = 12) ($P > 0.77$, two- tailed unpaired Student t test; $^{\#}P < 0.05$, one-sample t test compared with basal level.).

Synaptic destabilization and restabilization occur in the synapse after retraining stimulus in monosynaptic coculture

Because reactivation of the behavioral LTM for sensitization by retraining (two electrical shocks to the tail) also demonstrated labile and reconsolidation phases similar to reactivation of LTM by retrieval (brushing the siphon) (Lee et al., 2012), I investigated another reactivation method in sensory-to-motor neuron cocultures. I retreated the synaptic connection with five pulses of 5-HT to mimic the retraining protocol of electrical shocks used in the behavioral experiments. I found that five pulses of 5-HT increased the synaptic strength of sensory-to-motor neuron synapses at the second recording (Fig. 6). After the second recording 24 h later, I again applied five pulses of 5-HT as a reactivation (retraining) signal, then retested synaptic strength. Emetine treatment during and after 5-HT retreatment (5 × 5-HT + emetine) significantly disrupted the facilitation of the synapse, whereas retreatment with vehicle and 5-HT (5 × 5-HT + vehicle) did not affect maintenance of synaptic strength (Fig. 6). Moreover, βlac treatment combined with emetine (5 × 5-HT + βlac + emetine group) prevented emetine-induced disruption of LTF, whereas βlac treatment (5 × 5-HT + βlac) alone had no effect (Fig. 6). Results for the 5 × 5-HT + βlac + emetine group and the 5 × 5-HT + βlac group were not significantly different from those for the 5 × 5-HT + vehicle group at the third recording. Two-way ANOVA with time points (for the first and the third recordings) and drug groups as factors revealed significant effects of time points [$F_{(1,47)} = 21.98$, $P < 0.01$], drug groups [$F_{(3,47)} = 4.85$, $P < 0.01$], and the interaction between them [$F_{(3,47)} = 4.85$, $P < 0.01$]. Although we cannot exclude

the possibility that a single stimulation of the sensory neuron used to evoke the excitatory postsynaptic potential (EPSP) during the second recording provides sufficient activity to account for a partial effect of emetine (Fig. 3), this results do suggest that emetine has a stronger effect when applied in association with a multiple stimulation paradigm (HA) or 5-HT retraining that likely mimics stronger retrieval stimuli. Thus, once reactivated, LTF at the sensory-to-motor neuron synapse undergoes labile and reconsolidation phases.

Taken together, my data suggest that at the cellular levels, increased synaptic strength witch represent long-term memory becomes labile after reactivation or retraining signal, and that de novo protein synthesis-dependent reconsolidation is required to maintain long-term memory in the underling synaptic mechanism in the neural circuit of the reflex.

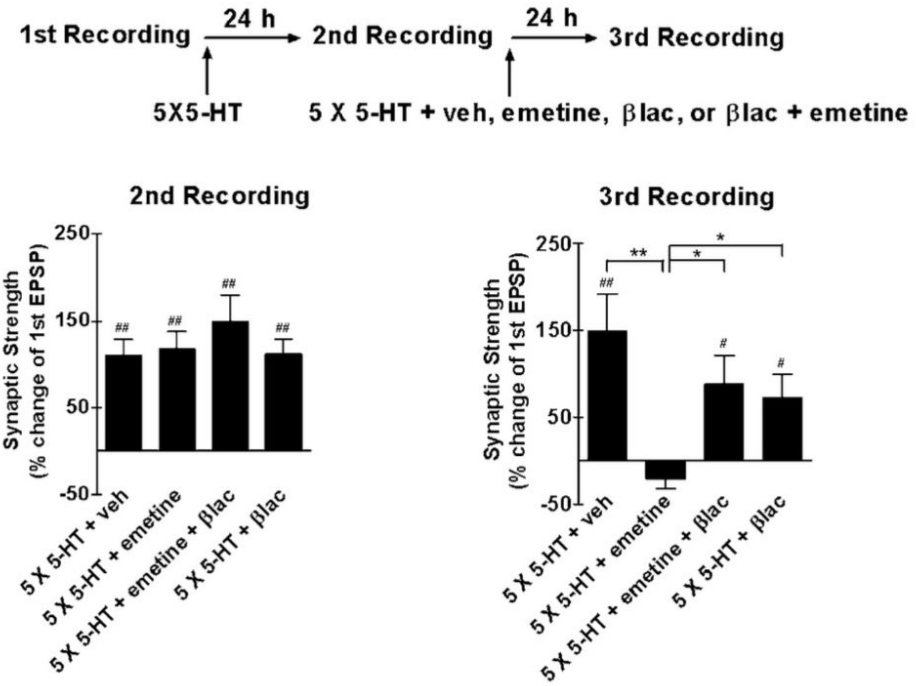

1st Recording
24 h
2nd Recording
24 h
3rd Recording
5X5-HT
5 X 5-HT + veh, emetine, βlac, or βlac + emetine
2nd Recording
Synaptic Strength
(% change of 1st EPSP)
250
150
50
-50
##
5 X 5-HT + veh
5 X 5-HT + emetine
5 X 5-HT + emetine + βlac
5 X 5-HT + βlac
3rd Recording
**
*
*
#

Figure 6. Synaptic destabilization and restabilization of LTF at the sensory-to-motor neuron synapse after reactivation by 5-HT treatment

(Upper) Schematic of the experimental procedure used to evaluate the effect of emetine on LTF after synaptic reactivation with 5-HT treatment.

(Lower) Bar graphs showing mean percentage change ± SEM in EPSP amplitudes. (Lower, Left) On the second recording, the changes in EPSP amplitudes were significantly different from the basal level recorded at the first recording, and were similar across groups. (Lower, Right) Concurrent application of emetine with five pulses of 5-HT (5 × 5-HT + emetine; n = 12) after the second recording impaired LTF at the third recording, whereas 5-HT (5 × 5-HT + veh; n = 12) or βlac treatment (5 × 5-HT + βlac; n = 14) alone after the second recording had no effect on LTF. βlac treatment combined with emetine (5 × 5-HT + βlac + emetine; n = 13) prevented LTF impairment on the third recording. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, one-sample t test compared with basal level [$F_{(3,47)} = 4.85$, $P < 0.01$; one-way ANOVA]. *P < 0.05, **P < 0.01, Newman–Keuls multiple- comparison test).

Fully consolidated synapse also requires new protein synthesis dependent reconsolidation for maintenance of LTF after retraining stimulus

A previous study found that the persistence of LTF in culture still requires local protein synthesis at the sensory-to-motor neuron synapse at 24 h and 48 h after treatment with five pulses of 5-HT, suggesting that the time window for consolidation is a natural consequence of long-term training (Miniaci et al., 2008a). Consequently, we tested whether de novo protein synthesis-dependent reconsolidation is also required for maintaining LTF at 72 h after the synaptic training—a time point at which inhibitors of local protein synthesis no longer disrupt the 5-HT–induced newly formed sensory neuron varicosities or LTF. The facilitated synapse was reactivated by five pulses of 5-HT at 72 h (retraining) after the first 5-HT treatment (training), and the strength was retested 48 h later (120 h after the training; Fig. 7), because the effect of emetine on both LTF and growth is more pronounced at this time point than at 24 h after its application (Miniaci et al., 2008a). Consistent with the results shown in Fig. 6, the inhibition of protein synthesis combined with synaptic reactivation impaired the facilitated synaptic strength, which returned to the basal level, whereas the facilitated synaptic strength induced by 5 × 5-HT treatment after the first recording was maintained in the vehicle and emetine groups (Fig. 8).

These data suggest that even at 72 h after the initial 5-HT training, when the facilitated synapse is quite stable (perhaps now more “fully consolidated”) and not disrupted by local application of inhibitors of protein synthesis, simple

reactivation of the sensory-to-motor neuron synapse can still induce the protein synthesis-dependent reconsolidation required to maintain the increase in synaptic strength. Although there is a possibility that our synaptic recording from 0 h to 48 h was obtained in a time window when the consolidation process was still in process, the data clearly show that memory storage, even in a partially consolidated state, can be further destabilized by a reactivating stimulus. Thus, even during earlier stages in the process of consolidation (at 24 h and 48 h after initial training), it is possible that reactivated synapses also may undergo a phase that requires new protein synthesis compared with non-reactivated synapses.

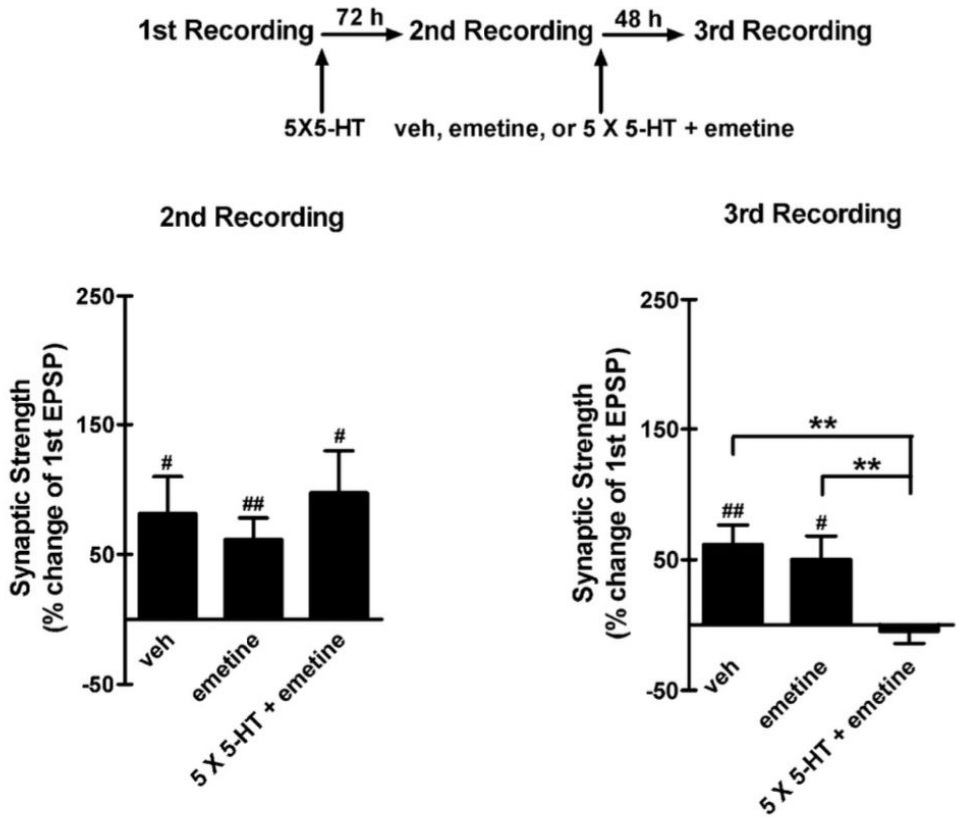

1st Recording
72 h
2nd Recording
48 h
3rd Recording
5X5-HT
veh, emetine, or 5 X 5-HT + emetine
2nd Recording
3rd Recording
Synaptic Strength
(% change of 1st EPSP)
250
150
50
-50
#
##
#
**
**
veh
emetine
5 X 5-HT + emetine

Figure 7. Protein synthesis is required for maintaining LTF at the sensory-to-motor synapse reactivated by 5-HT treatment at 72 h after training

(Upper) Schematic of the experimental procedure used to evaluate the effect of emetine on LTF after synaptic reactivation with 5-HT treatment.

(Lower) Bar graphs showing the mean percentage change ± SEM in EPSP amplitudes.

(Lower, Left) On the second recording, the changes in EPSP amplitudes were significantly different from the basal level recorded at the first recording, and were similar across groups.

(Lower, Right) Concurrent application of emetine with five pulses of 5-HT (5 × 5-HT + emetine; n = 10) at 72 h after the first 5-HT treatment (training) impaired LTF at 120 h after the training, whereas treatment with vehicle alone (veh; n = 9) or emetine alone (emetine; n = 7) had no effect on LTF. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, one-sample t test compared with basal level [$F_{(2,23)} = 7.52$, $P < 0.01$, one-way ANOVA]. **P < 0.01; Newman–Keuls multiple-comparison test.

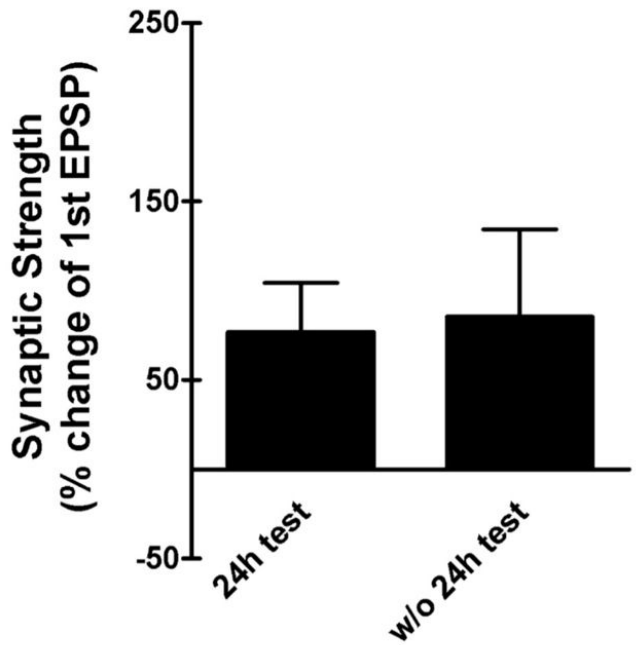


Figure 8. Effect of synaptic strength test at 24hr on EPSP recording at 72hr

Synaptic strength measured at 72 h after the first 5-HT treatment (training) with (24 h test; n = 4) or without (w/o 24 h test; n = 5) testing of synaptic strength at 24 h after the training. There was no difference between the two test groups ($P > 0.89$, two-tailed unpaired Student t test). Data are from the vehicle group (n = 9) shown in previous Figure 7.

DISCUSSION

Our results suggest that sensory-to-motor neuron synapses, the primary components of the neural circuit underlying behavioral sensitization, are destabilized by means of ubiquitin/proteasome-dependent protein degradation after memory retrieval or retraining and are restabilized by a protein synthesis-dependent reconsolidation process. These results demonstrate that, at least in this model learning system, reconsolidation after memory retrieval or retraining involves transient and regulated changes of the stored memory trace at the same synaptic connections that were initially modified for storage of that trace. Furthermore, these synaptic mechanisms in *Aplysia* are likely to share important similarities with those that underlie reconsolidation in the mammalian brain, because many molecular mechanisms of learning and memory storage are shared by invertebrates and vertebrates.

The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (Hegde et al., 1993; 1997) and FMRFamide-induced depression (Fioravante et al., 2008). The ubiquitin/proteasome system also functions as an inhibitory constraint on synaptic strength and growth in a translation-dependent, but not a transcription-dependent, manner (Zhao et al., 2003). Indeed, consistent with results of Zhao et al. (Zhao et al., 2003), we also observed an increase of

both basal synaptic transmission at sensory-to-motor neuron synapses in culture in the presence of higher concentrations (1 μM) of βlac. However, the treatment of βlac at lower concentrations (0.1 μM for synapse recording) affected only the reconsolidation and had no affect on the basal level or consolidation process in cultured synapses (Fig. 4 and Fig. 5). These results suggest that compared with the consolidation process, the reconsolidation process may be more sensitive to inhibition of the ubiquitin/proteasome system.

It has been reported that synapse in the amygdala and hippocampus undergo a labile phase initated by reactivation (Lee et al., 2008a; Kim et al., 2010). However, complexity by multiple synaptic connections in mammalian system was not possible to test whether synaptic destabilization and restabilization occurred at the same synapses that initially encoded the memory. This study provide the first direct evidence that the same specific synaptic connections that initially encode the stored memories are selectively destabilized and restabilized after memory retrieval. This is consistent with the idea that reconsolidation represents a continuation of the consolidation process at the same set of synaptic connections and may serve to strengthen memory storage by allowing it to become longer-lasting and more stable. Whether or not reconsolidation in *Aplysia* also may represent an updating process in which the synapses that encode the preexisting memory are further reorganized after memory retrieval so as to recruit new synaptic connections that allow the incorporation of new information was not addressed in this study. Future imaging studies in *Aplysia* cultures using bifurcated

sensory neuron where the “circuit” of a sensory neuron is expanded by the addition of more than one type of target cell may help answer this question.

The simplicity of the model system used in this study help to answer a set of important questions to further understand the function of reconsolidation. For example, Does the process of reconsolidation recruit some of the same cellular and molecular mechanisms that underlie consolidation? Do ApCPEB4 and ApCPEB, which are essential for the initiation and maintenance of LTM in *Aplysia*, also play a pivotal role in the reconsolidation-dependent strengthening of memory storage? What is degraded and which proteins are resynthesized during the reorganization process? Is this process occurring only in presynaptic? or also in post-synaptic? Answers to these questions with further study will provide better understanding for the role of reconsolidation in long-term memory process.

CHAPTER III

ApCPEB4-like protein, a non-prion domain containing homolog of ApCPEB, is involved in the initiation of long-term facilitation

INTRODUCTION

Unlike short-term memory, long-term memory requires new protein synthesis for its formation (Rainbow, 1979; Sweatt and Kandel, 1989; Pedreira et al., 1995; Lee et al., 2008b). Protein synthesis occurs in two spatially distinct regions of the neuron: 1) in the cell body where activity-dependent transcription and subsequent translation occurs and 2) in the postsynaptic dendritic spines and in the presynaptic terminal, where mRNAs are localized and translated following synaptic activation (Martin et al., 1997b; Schuman et al., 2006; Lebeau et al., 2011). The second form of translation is responsible for local protein synthesis, which is important for both the initiation and the maintenance of long-term memory.

The cytoplasmic polyadenylation element binding protein (CPEB) has been identified as one key regulator of the local protein synthesis in *Aplysia*. The binding of CPEB to mRNAs regulates the translation of target mRNAs by regulating the polyadenylation (Wells et al., 2000; Richter, 2001; Kim and Richter, 2006; Richter, 2007). ApCPEB binds to the 3' untranslated region (3' UTR) of mRNAs that contains conserved cytoplasmic polyadenylation element (CPE) site (UUUUUAU) (Si et al., 2003b). ApCPEB is locally activated in response to a single pulse of 5-hydroxytryptamine (5-HT) and is inhibited by rapamycin. Interestingly, ApCPEB has a prion-like domain that is important for maintaining the increased level of ApCPEB proteins, and thereby critical for maintaining long-term facilitation (LTF) in *Aplysia* sensory-motor neuron synapse (Si et al., 2003d;

2010b). When the translation of the ApCPEB mRNA is blocked locally, the initiation of LTF at 24 hrs is intact whereas the maintenance of LTF at 72 hrs is specifically impaired. One of the major mRNA targets of ApCPEB is the actin mRNA, which contains the CPE site on its 3' untranslated region (3' UTR) and is locally translated during LTF (Si et al., 2003b). ApCPEB has two isoforms, one contains poly-Q domain and the other lacking the prion-like domain (Liu and Schwartz, 2003a; Si et al., 2003b). The maintenance of LTF requires the long form of ApCPEB, indicating that the poly-Q domain mediated stabilization of ApCPEB is important for the activation of ApCPEB signaling.

In this study, we cloned a new CPEB homolog, ApCPEB4-like protein, in *Aplysia kurodai*. The level of expression of ApCPEB4 was increased by 5-HT in a translation dependent manner. Unlike ApCPEB, ApCPEB4 bound to specific RNA in a CPE- independent manner and is required for the initiation but not for the maintenance of LTF. Overexpression of ApCPEB4 reduced the threshold of the LTF induction. In addition, PKA- mediated phosphorylation of ApCPEB4 was critical for the induction of LTF. Collectively, these data suggest that ApCPEB4 plays a key role in regulating the formation of LTF, while ApCPEB is essential for the maintenance of LTF.

EXPERIMENTAL PROCEDURES

Cloning of ApCPEB4 from *Aplysia kurodai*

We obtained the ApCPEB4 fragment of *Aplysia kurodai* from EST database by searching through custom-made basic local alignment software. Using this fragment as a probe, we screened ~1.5 X 10^5 clones of an *Aplysia kurodai* cDNA library and isolated several clones encoding parts of ApCPEB4. Based on the sequences of these clones, we obtained the full length of ApCPEB4. The length of coding region was 2064 bp and 664 amino acids, and it also contained two RNA Recognition Motifs (Fig. 9A). Using Expasy software (www.expasy.org), potential PKA phosphorylation sites were searched.

3X CPE or CPE mutant sites were obtained by PCR with specific primer sets:

3xCPE1,

CPE1-D3-S (5'-CGCCCAAGCTTGCAGCTTTTTATGACACACAGTTTTTATGATGCCACG-3')/

CPE1-EI-A (5'-GCATGAATTCGATGGATAAAAACGTGGCACATAAAAACTGTGTGTC-3');

3x CPE2,

CPE2-D3-S (5'-CGCCCAAGCTTGCAGCTTTTAATGACACACAGTTTTAATGATGCCACG-3')/

CPE2-EI-A (5'-GCATGAATTCGATGGATTAAAACGTGGCATCATTAAAACTGTGTGTC-3');

3x CPE3,

CPE3-D3-S (5'-CGCCCAAGCTTGCAGCTTTTATAAGGACACACAGTTTTATAAGGATGCCACG-3')/

CPE3-EI-A (5'-GCATGAATTCGATGGCTTATAAAACGTGGCATCCTTATAAAACTGTGTGTC-3');

3x CPEmt1,

CPEmt1-D3-S (5'-CGCCCAAGCTTGCAGCTTTTTGTG ACACACAGTTTTTGTGATGCCACG-3')/

CPEmt1-EI-A (5'-GCATGAATTCGATGGACAAAAACGTGGCATCACAAAAACTGTGTGTC-3');

3x CPEmt2,

CPEmt2-D3-S (5'-CGCCCAAGCTTGCAGCTTTTTGGTGACACACAGTTTTTGGTGATGCCACG-3')/

CPEmt2-EI-A (5'-GCATGAATTCGATGGACCAAAAACGTGGCATCACCAAAAACTGTGTGTC-3').

The PCR products were separately sub-cloned into *Hind*III–*Eco*RI-digested pcDNA3.1(+) to create pcDNA3.1-3xCPEs.

Kinase assays

A kinase assay was carried out at 30 °C for 30 minutes in a final volume of 25 μl of reaction buffer (50 mM Tris-Cl, 10 mM $MgCl_2$, pH 7.5) containing 1 μg substrate, 200 μM ATP, 1 mCi [γ^{32}P]ATP and 5 units of PKA catalytic subunit (NEB). Reactions were stopped by adding SDS-sample buffer and boiling at 100 °C for 5 minutes. Then, [^{32}P] phosphate incorporation was analyzed by SDS-PAGE and a phosphoimager. To confirm the specificity of phosphorylation by PKA, either 40 μM KT5720 (AG Science) or dimethyl sulfoxide (DMSO) (Sigma) was added to the reaction mixture.

To examine whether ApCPEB4 is an endogenous substrate of *Aplysia* PKA, the crude tissue extract from *Aplysia* pedal-pleural ganglia was prepared as previously described(Yamamoto et al., 1999). The reaction was carried out at 18 °C for 20 minutes containing GST-agarose bead binding 1 μg of GST-ApCPEB4, 10 μg of tissue extract and 1 mCi [γ^{32}P]ATP in extraction buffer. To confirm the specificity of phosphorylation, the crude tissue extracts were incubated with inhibitors of specific kinases, 40 μM KT5720 (PKA inhibitor)(Khabour et al., 2004), 20 μM PD98059 (MEK inhibitor) or 10 μM chelerythrin (PKC inhibitor), for 10 minutes. A GST-pull down assay was performed as previously described(Sander et al., 1998). The [^{32}P] phosphate incorporation was analyzed by

SDS-PAGE and a phosphoimager.

Recombinant protein purification and antibody production

For the antibody production, the N-terminal 400bp of ApCPEB4 was amplified by PCR and ligated into pRSETa (Invitrogen), a His-tag vector. The His-ApCPEB4-N protein expression was induced by 2 mM IPTG for 3 hrs at 37°C and purified by a Ni-NTA purification system (Invitrogen). Polyclonal anti-ApCPEB4 antibodies were raised in mice using this purified protein. The peptide competition assay was performed by western blot using the ApCPEB4 antibodies incubated with either 25 μg of purified His-ApCPEB4-N or 25 μg of BSA as a control at 4°C overnight.

RT-PCR, western blot, and immunocytochemistry

To examine the expression of ApCPEB4, an RT-PCR was performed using the total RNAs from various *Aplysia* tissues or HEK293T cells using gene-specific primers. For loading control, PCR was performed against S4 for *Aplysia*. For the induction control, PCR was performed against *Aplysia* CCAAT-enhancer-binding proteins (ApC/EBP). A western blot was performed in the pleural ganglia, buccal muscle, and gill extracts. Anti-ApCPEB4, and anti-actin antibodies were used to detect each protein within the same loaded sample. To examine the induction level of ApCPEB4 in response to 5-HT, pleural-pedal ganglia were prepared in a sylgard plate and then applied with 5 pulses of 5-HT (20μM for 5min at 20 min interval). Pleural ganglia were prepared 30min after final application of 5-HT. For the immunostaining of endogenous ApCPEB4, cultured neurons were

washed with cold ASW twice and immediately fixed with 4% paraformaldehyde in PBS after either the application of massed 5-HT (10μM for 1 hr) or 5 pulses 5-HT (10μM for 5 min) at 20min interval. Fixed cells were washed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 10 min. After blocking with 3% BSA (Amersham Biosciences, Piscataway, NJ) for 2 hrs at room temperature, primary antibodies were treated (1:500 of anti-ApCPEB4 serum) overnight at 4℃. The cells were washed with PBS and treated with secondary antibody, Cy3-conjugated anti-mouse IgG (Amersham Biosciences, Piscataway, NJ) for 1 hr at room temperature. Immunostained images were acquired by a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany).

mRNA-protein pull-down assay

mRNA-protein pull-down assay was performed as described previously(Mastushita-Sakai et al., 2010) with small modification. Actin 3' UTR was obtained from *Aplysia* ganglion cDNA, and Luciferase-1904 (Luc-1904) was obtained by oligomer annealing and subcloned into pGL3UC vector (Promega)(Huang et al., 2006b). The biotin labeled RNA was prepared by *in-vitro* transcription with T7 RNA polymerase (Promega) using the nucleotide analog Bio-17-ATP and Bio-11-CTP (Enzo). Each biotinylated RNA was analyzed by agarose-gel electrophoresis and quantified by nano-drop. HEK293T cells overexpressing Flag-tagged target proteins were lysed using lysis & binding buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5% glycerol, 0.1% Triton X-100, 1 mM DTT, 0.2 mg/mL heparin, 0.2 mg/mL yeast tRNA, 0.25% BSA, protease inhibitor cocktail (Roche), and 40 U/mL RNasin (Promega). 8 μg of biotinylated RNAs

were mixed with pre-cleared 200 μg (0.2 mg/mL) of 293T cell lysate and incubated on a rotator for 1 hr at 4 ^{o}C. 30 μl of NeutraAvidin Agarose Resin (Thermo) was added to each tube, and the mixture was further incubated for 2 hrs. Beads were washed five times with washing buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5% glycerol, 0.1% Triton X-100, 1 mM DTT and 40 U/mL RNasin. Western blots were performed with mFlag-M2 antibody (1:2000, Sigma).

Cell cultures and microinjection

Primary culture of *Aplysia* sensory neurons and coculture of sensory-to-motor neurons were made as described previously(Montarolo et al., 1986; Lee et al., 2001; 2003). Briefly, Abdominal and central ganglia were dissected from *Aplysia kurodai* (50-100g) and incubated at 34 °C for 1.5~2.5 hr in 1 % protease (type IX, Sigma) dissolved in isotonic L15/ASW (1:1) media (ASW: 460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, and 10 mM HEPES, pH 7.6). After a thorough washing with ASW several times to remove residual protease, the ganglia were incubated at 18 ^{o}C for at least 3 hrs in L15/ASW to allow for recovery from heat shock. LFS motor neurons were dissected from the abdominal ganglia and cultured in a solution of 50 % *Aplysia* hemolymph in isotonic L15 media. The next day, pleural sensory neurons were isolated from the pleural ganglia and cocultured with LFS motor neurons and maintained at 18 °C in an incubator for 3 days to allow time for the formation and stabilization of synaptic connections. Microinjection of DNAs and double-strand RNAs into *Aplysia* neurons was done by PLI-100 Pico-injector (Medical systems).

Electrophysiology

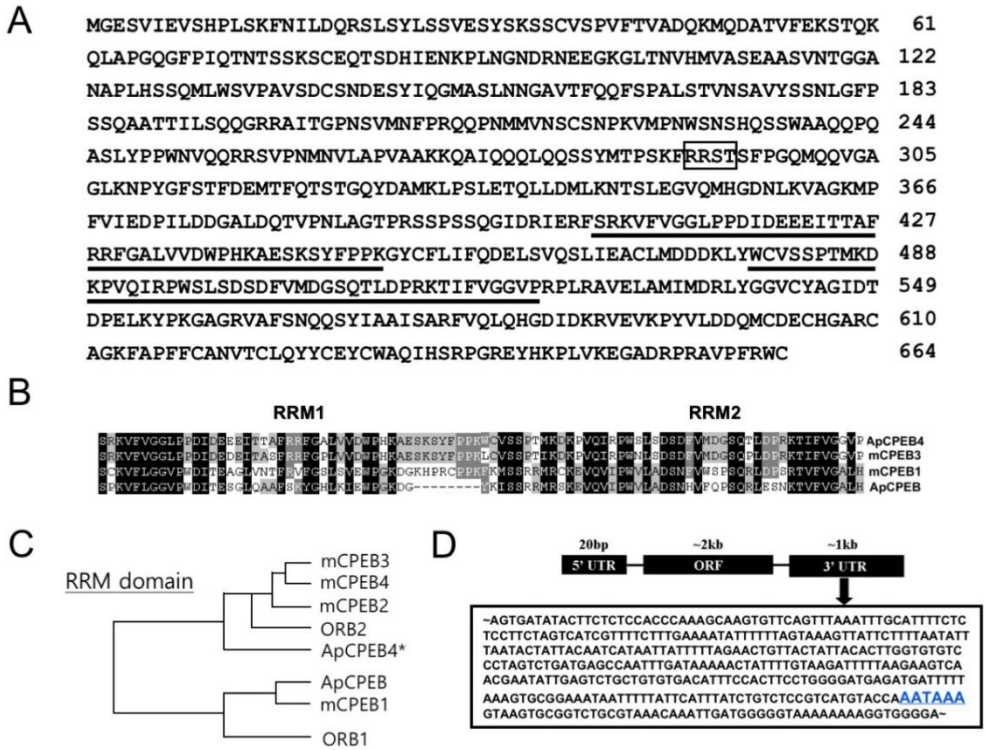
The LFS motor neuron was impaled with a glass microelectrode filled with 2 M K-acetate, 0.5 M KCl, 10 mM K-HEPES (10-15 MΩ), and the membrane potential was held at –80 mV. The excitatory postsynaptic potential (EPSP) in the motor neuron was evoked by stimulating the sensory neurons with a brief depolarizing stimulus using an extracellular electrode. The initial EPSP value was measured 24 hrs after microinjection. The cultures then received one pulse or five pulses of 10 μM 5-HT for 5 minutes at 15-min interval to induce LTF. The amount of synaptic facilitation was calculated as a percentage change in EPSP amplitude recorded after the 5-HT treatment compared with its initial value before treatment.

RESULT

Cloning of ApCPEB4-like protein, a homologue of *Aplysia* CPEB

As an initial step for investigating the role of other CPEBs in *Aplysia*, We obtained an expressed sequence tag (EST) clone homologous to the conserved RNA recognition motif (RRM) of mammalian CPEB2-4 family from the *Aplysia kurodai* EST database (Choi et al., 2010). Using this EST clone as a probe, we carried out a library screening and cloned a full-length cDNA of a novel *Aplysia* CPEB (Fig. 9A). We named the clone ApCPEB4 as it is 99% identical to CPEB4-like gene in the genomic database of *A. californica* (NCBI accession #, XP005089812). ApCPEB4 has a unique N-terminus and two conserved RRM on the C- terminus (Theis et al., 2003; Si et al., 2003b) (Fig. 9A). Unlike the long form of ApCPEB, which was cloned previously (Liu and Schwartz, 2003b), ApCPEB4 does not have poly-Q domain. ApCPEB4 has a potential PKA phosphorylation site (RRST, consensus sequence (RRX(S/T)) outside the RRM domains (Fig. 9A). Even though the sequence was not identical, the overall phylogenetic analysis of the phosphorylation site and the RRM domain of ApCPEB4 revealed that ApCPEB4 is homologous to mammalian CPEB2-4 and Orb2 (Fig. 9B and 12C). The amino acid sequences of the ApCPEB4 RRM domain are 83.0 % identical to mouse CPEB2, 82.0 % to mouse CPEB3, 80.7 % to mouse CPEB4, 77.4 % to Orb2, 34.4 % to mouse CPEB1, 32.7 to Orb1 and 31.0 % to

ApCPEB, respectively. These analyses suggest that ApCPEB4 belongs to mammalian CPEB2-4 family. Interestingly, the ApCPEB4 3' untranslated region (UTR) (~1kb) contains the nuclear polyadenylation hexanucleotide sequence (Fig. 9D). We next examined the expression of ApCPEB4 in various *Aplysia* tissues by Reverse Transcription-Polymerase Chain reaction analysis (RT-PCR). ApCPEB4 was expressed in the extracts of central nervous system (CNS) and other tissues including gill and ovotestis (Fig. 10A). Western blot analysis detected significant bands with the size of ~100kDa and ~70kDa in both purified proteins and protein extracts from *Aplysia* pleural ganglia, respectively (Fig. 10B). Taken together, these data indicate that ApCPEB4 is another neuronal CPEB protein that belongs to CPEB2-4 family in *Aplysia*.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SSQAATTILSQQGRRAITGPNSVMNFPRQQPNMMVNSCSNPKVMPNWSNSHQSSWAAQQPQ 244
ASLYPPWNVQQRRSVPNMNVLAPVAAKKQAIQQQLQQSSYMTPSKFRRSTSFPGQMQQVGA 305
GLKNPYGFSTFDEMTFQTSTGQYDAMKLPSLETQLLDMLKNTSLEGVQMHGDNLKVAGKMP 366
FVIEDPILDDGALDQTVPNLAGTPRSSPSSQGIDRIERFSRKVFVGGLPPDIDEEEITTAF 427
RRFGALVVDWPHKAESKSYFPPKGYCFLIFQDELSVQSLIEACLMDDDKLYWCVSSPTMKD 488
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DPELKYPKGAGRVAFSNQQSYIAAISARFVQLQHGDIDKRVEVKPYVLDDQMCDECHGARC 610
AGKFAPFFCANVTCLQYYCEYCWAQIHSRPGREYHKPLVKEGADRPRAVPFRWC 664
B
RRM1
RRM2
ApCPEB4
mCPEB3
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~AGTGATATACTTCTCTCCACCCAAAGCAAGTGTTCAGTTTAAATTTGCATTTTCTC
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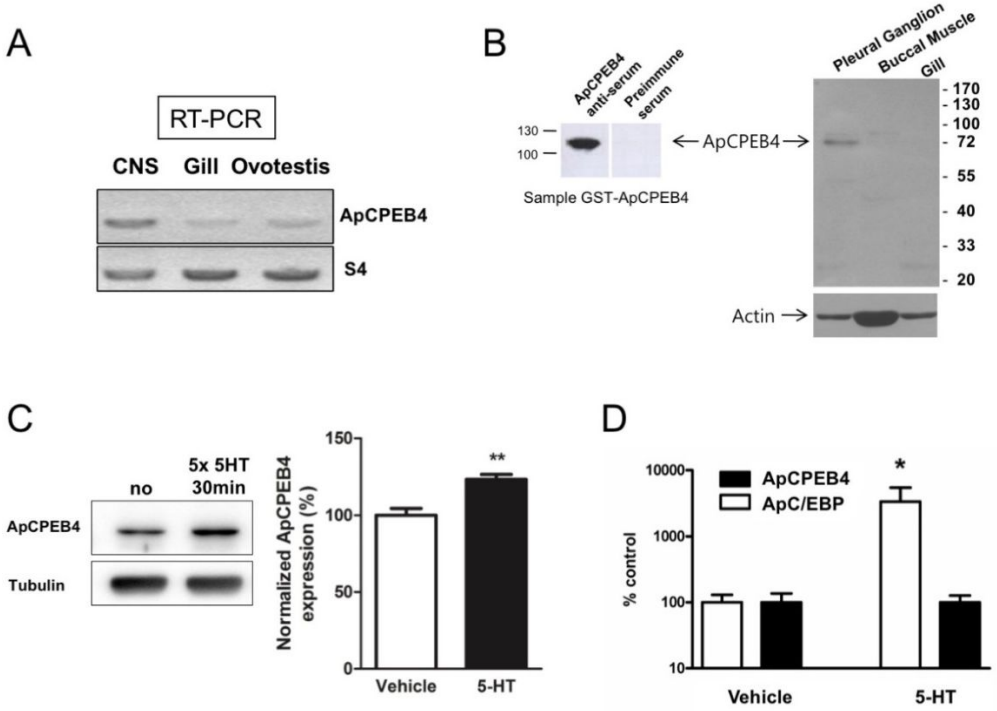
GTAAGTGCGGTCTGCGTAAACAAATTGATGGGGGTAAAAAAAAGGTGGGGA~

Figure 9. Cloning of ApCPEB4 and its expression in the CNS

(A)Amino acid sequence of a cloned full-length ApCPEB4. Sequence analysis showed that ApCPEB4 had two conserved RRMs (underlined), one conserved PKA phosphorylation sites (box). (B) Alignment of RRM domain of *Aplysia* CPEB4 (ApCPEB4), mouse CPEB3 (mCPEB3), mouse CPEB1 (mCPEB1) and *Aplysia* CPEB (ApCPEB). (C) The phylogenetic relationship between CPEBs in different species was determined by ClustalW. (D) mRNA structure of the ApCPEB4. ApCPEB4 contains ~20 bp 5' UTR (untranslated region), ~2 kb open reading frame (ORF), and ~1kb 3' UTR. Arrowed inset indicates the detailed nucleotide sequence of the 3' UTR. Blue underline indicates hexanucleotide sequence (AATAAA).


A
RT-PCR
CNS
Gill
Ovotestis
ApCPEB4
S4
B
ApCPEB4 anti-serum
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100
Sample GST-ApCPEB4
ApCPEB4
Pleural Ganglion
Buccal Muscle
Gill
170
130
100
72
55
40
33
20
Actin
C
no
5x 5HT 30min
ApCPEB4
Tubulin
Normalized ApCPEB4 expression (%)
150
100
50
0
**
Vehicle
5-HT
D
ApCPEB4
ApC/EBP
% control
10000
1000
100
10
*
Vehicle
5-HT

Figure 10. Expression of ApCPEB4 in the CNS

(in collaboration with Nam-Kyung Yu and Chae-Seok Lim)

(A) Expression pattern of ApCPEB4. RT-PCR of total RNA (1 μg) isolated from *Aplysia* CNS, ovotestis, or gill with gene-specific primers. *Aplysia* housekeeping gene S4 was used as a control for the amplification. (B) Western blotting of ApCPEB4 using purified GST-fused ApCPEB4 or total lysates from various tissues including pleural ganglion, buccal ganglion and ovotestis. (C) A representative Western blot (left) and quantification (right) of ApCPEB4 in *Aplysia* pleural ganglia extracts prepared from pleural-to-pedal ganglia exposed to 5 times of 5 min treatment of 5-HT. Total extracts were prepared at indicated times and 20 μg of proteins were blotted with anti-ApCPEB4 antibodies (left, top panel). The same extracts were also blotted with anti-tubulin antibodies as loading controls (left, bottom panel). 5-HT treatment significantly increased the level of ApCPEB4 in the extracts. **, $p < 0.01$ (D) One microgram of total RNA from pleural ganglia was used for RT-PCR with gene-specific primers. As a stimulation control, we used ApC/EBP, an immediate early gene. ApC/EBP was transcriptionally enhanced in response to 5-HT stimuli. *Aplysia* S4 was used as an amplification and loading control. *, $p < 0.05$ compared to that of control ApC/EBP.

ApCPEB4 is synthesized in response to 5-HT signaling

Next question I asked was whether the expression of ApCPEB4 is regulated in response to 5-HT signaling. I found that the level of ApCPEB4 protein in the ganglia extracts was significantly increased by either repeated (5 times, 5 min each) (Fig. 10C) or massed (2 hrs) (Fig. 11) application of 5-HT onto the intact pleural-to-pedal ganglia, both of which are known to induce long-term facilitation. The increase in protein level was not transcription-dependent, because ApCPEB4 RNA transcript was not increased by 5-HT treatment (Fig. 10D).

Transcription-independent increase of ApCPEB4 suggests that 5-HT signaling may regulate translation of ApCPEB4 mRNA or stability of ApCPEB4 protein. I first examined whether ApCPEB4 mRNA was localized at the distal neurite. When the 3' UTR of ApCPEB4 was added at the end of the cDNA sequence of the reporter gene nGFP (nuclear GFP), the GFP signal was observed at the distal neurite (Fig. 12A). This supports the idea that the 3' UTR of ApCPEB4 is sufficient for the localization and translation of the mRNA at the distal neurite. Next, I cut off the cell bodies of cultured sensory neurons, and stimulated the isolated neurites for 1 hr with 10 μM 5-HT. ApCPEB4 immunoreactivity was increased about 2 fold in the stimulated neurites compared with that of the vehicle-treated neurites (vehicle, 100.0 ± 14.4 %, n = 6 versus 5-HT, 186.8 ± 17.8 %, n = 6; t-test, ** $p < 0.01$) (Fig. 12B). This increase is also observed in the neurites treated with pulsed 5-HT (5 min of 10 μM 5-HT, 5 times; vehicle, 100.0 ± 46.1 %, n = 43 versus 5X5-HT, 128.8 ± 5.9 %, n = 60; t-test, ** $p < 0.01$). The up-regulation of ApCPEB4 was blocked by emetine (100 μM), a non-selective protein synthesis

inhibitor (vehicle, 100.0 ± 14.4 %, n = 6; 5-HT, 186.8 ± 17.8 %, n = 6; emetine, 98.24 ± 26.9 %, n = 5; t-test, ** $p < 0.01$, * $p < 0.05$) (Fig. 12B). Conversely, the induction of ApCPEB4 was not affected by the transcriptional inhibitor, actinomycin D (50 μM) (actD, 244.3 ± 20.7 %, n = 7; t-test, $p > 0.05$) (Fig. 12B). These results together suggest that 5-HT signaling enhances translation, but not transcription of ApCPEB4 mRNA in the stimulated neurites.

Two distinct translational mechanisms are known to be recruited during 5-HT- mediated synaptic facilitation in *Aplysia*: rapamycin-sensitive and -insensitive ones (Casadio et al., 1999). Since ApCPEB4 was translated in the isolated neurites, I further tested whether this translational induction is sensitive to the rapamycin. When the rapamycin (20 nM) was added together with 5-HT on the isolated neurites, translational induction of ApCPEB4 was not blocked, indicating that translation of ApCPEB4 is rapamycin-insensitive (Fig. 12C). Because rapamycin-insensitive, but emetine-sensitive local translation requires protein kinase A (PKA) activity for the initiation of synapse-specific LTF (Martin et al., 1997b; Casadio et al., 1999), I tested whether local translation of ApCPEB4 is PKA dependent. Translation of ApCPEB4 was blocked by KT-5720 (PKA inhibitor, 5 μM) (vehicle, 100.0 ± 2.5 %, n = 10 versus 5-HT, 150.4 ± 13.7 %, n = 14; t-test ** $p < 0.01$; KT-5720, 86.4 ± 10.8 %, n = 13; t-test ** $p < 0.01$; rapamycin, 146.7 ± 9.5 %, n = 13; t-test $p > 0.1$) (Fig. 12C), raising the possibility that the translation of ApCPEB4 might be critical for the initiation of LTF.

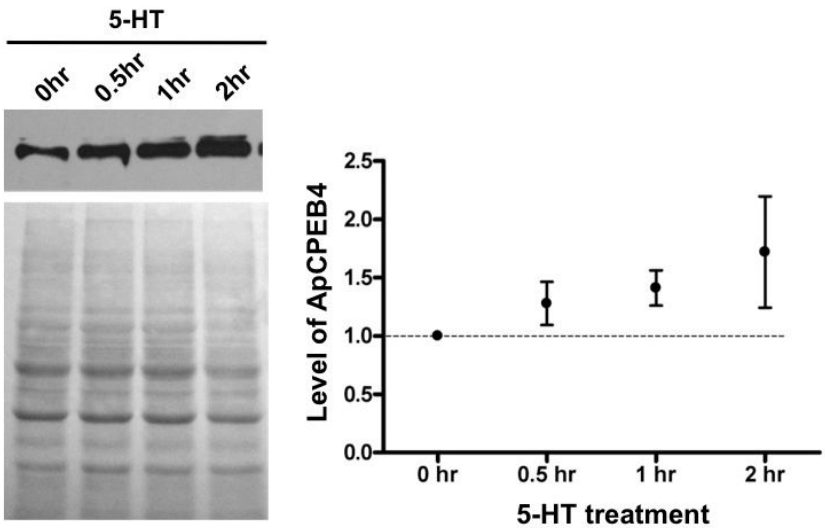
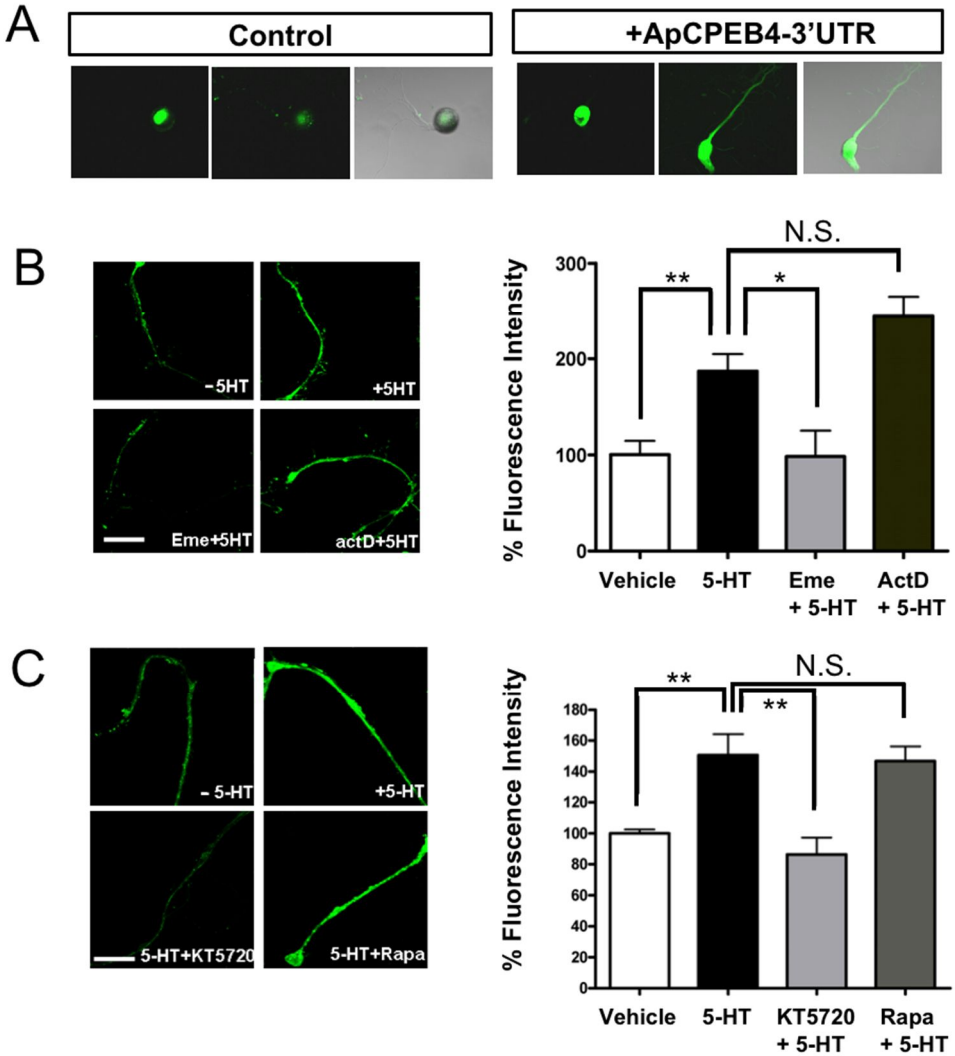


Figure 11. Expression of ApCPEB4 is increased by 5-HT treatment in vivo

A representative Western blot (left) and quantification (right) of ApCPEB4 in *Aplysia* pleural ganglia extracts prepared from animals exposed to 5-HT *in vivo* for 2 hrs. Total extracts were prepared at indicated times and 20 μg of proteins were blotted with anti-ApCPEB4 antibodies (left, top panel). The same extracts were also stained with Coomassie blue as loading controls (left, bottom panel).


A
Control
+ApCPEB4-3'UTR
B
-5HT
+5HT
Eme+5HT
actD+5HT
% Fluorescence Intensity
N.S.
**
*
300
200
100
0
Vehicle
5-HT
Eme + 5-HT
ActD + 5-HT
C
- 5-HT
+5-HT
5-HT+KT5720
5-HT+Rapa
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N.S.
**
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180
160
140
120
100
80
60
40
20
0
Vehicle
5-HT
KT5720 + 5-HT
Rapa + 5-HT

Figure 12. ApCPEB4 expression is increased by the activation of 5-HT signaling in the isolated neurites *(in collaboration with Seung-Hee Lee)*

(A) ApCPEB4 3' UTR enhances local translation of reporter cDNA. The reporter gene nGFP (nuclear GFP) expression, which normally occurs in the nucleus (control), was observed at the distal neurite in the presence of the 3' UTR of ApCPEB4 (+ApCPEB4- 3' UTR).

(B) Immunostaining for ApCPEB4 showed significant induction of ApCPEB4 following 5-HT application in the isolated neurites. The induction of ApCPEB4 was blocked by concurrent treatment of emetine, not by actinomycin D (actD).

(C) Concurrent treatment of KT5720, a PKA inhibitor, significantly blocked the induction of ApCPEB4 following 5- HT treatment, while the rapamycin (rapa), a blocker for mTOR-dependent protein translation, has no effect on the ApCPEB4 induction. *, $p< 0.05$; **, $p<0.01$; n.s., not significant.

RNA binding specificity of ApCPEB4

A growing body of evidence suggests that mammalian CPEB1 and CPEB2-4 family have different target RNAs. For example, CPEB1 has higher affinity to CPE site on the 3' UTR of target mRNAs, but CPEB3 and CPEB4 are believed to recognize specific RNA secondary structure (Huang et al., 2006a). We tested whether *Aplysia* CPEB proteins, ApCPEB and ApCPEB4, also show difference in RNA binding properties. We first generated five different target RNA constructs containing three types of three-repeated (3X) CPE sites (CPE1 (UUUUUAU), CPE2 (UUUUAUU) and CPE3 (UUUUAUAAG) or two types of 3X CPE mutant sites (CPEmt1 (UUUUUGU) and CPEmt2 (UUUUUGGU)) (Fig. 13A). ApCPEB4 did not bind to any CPE or CPE mutant site, whereas ApCPEB bound to CPE sites but not to CPE mutant sites (Fig. 13B). These results indicate that ApCPEB4 and ApCPEB have different RNA binding properties. We further tested this idea by using the CPE site of the 3' UTR of *Aplysia* actin, which is a target mRNA of the ApCPEB (Si et al., 2003d). Interestingly, ApCPEB4 did not bind to CPE site in the 3' UTR of *Aplysia* actin, which contains a well-known CPE site (UGUAUUUUUUAUACAAUGUU), whereas ApCPEB showed specific binding to it (Fig. 13C). Instead, ApCPEB4 bound to SELEX 1904 U-rich sequence (AAAGAGGAUUUGUGUUUUUCAGGAC), which was designed with SELEX(Systematic evolution of ligands by exponential enrichment) analysis as a target mRNA for mammalian CPEB3 and CPEB4 (Huang et al., 2006a) (Fig. 13C). These results suggest that ApCPEB4 is similar to mammalian CPEB3-4 family in its RNA-binding properties. Overall, these results suggest that ApCPEB4 is functionally closer to the mammalian CPEB3-4 family and is different from ApCPEB in its target selectivity.

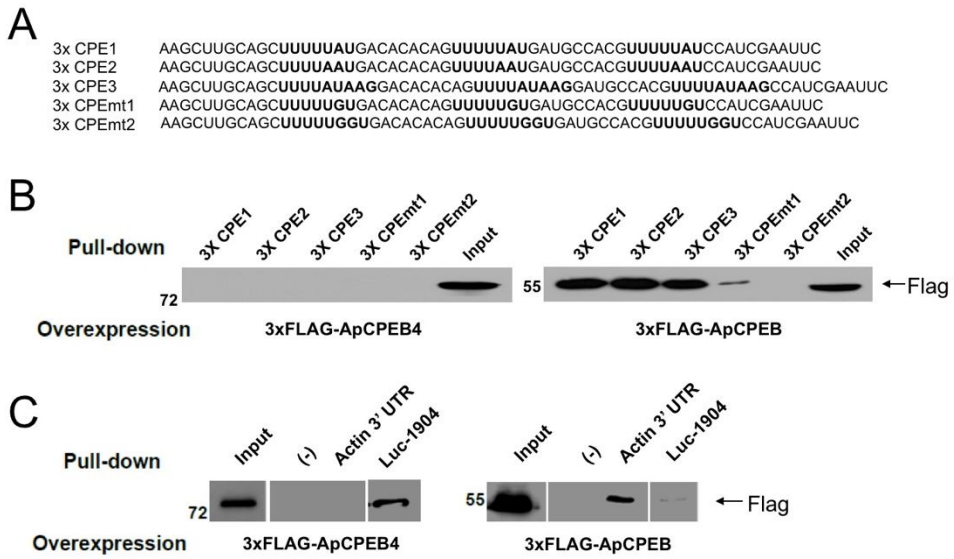


Figure 13. RNA binding specificity of ApCPEB4 and ApCPEB

(in collaboration with Deok-Jin Jang)

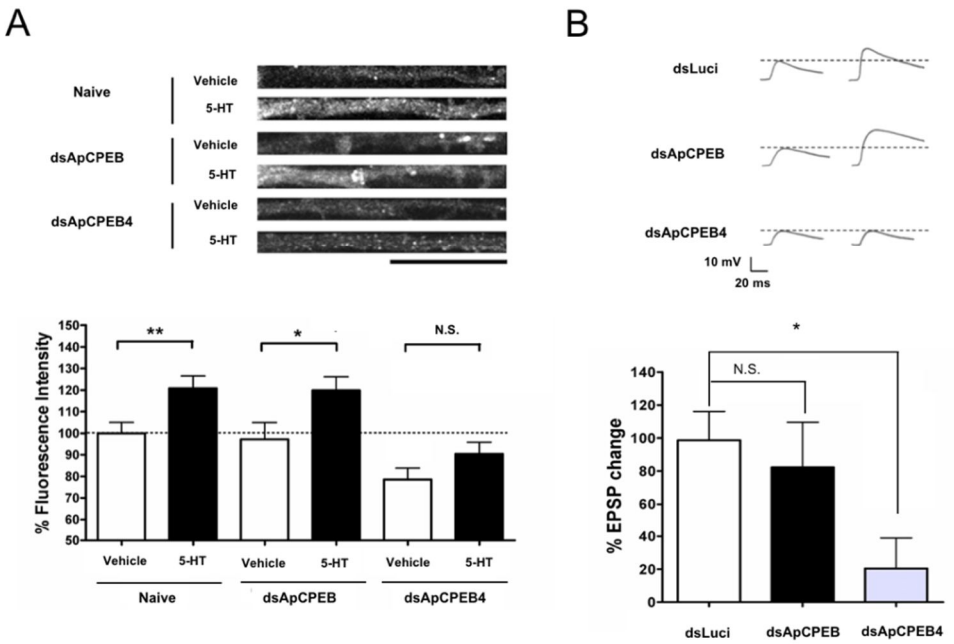
(A) RNA sequences of CPE1, CPE2, CPE3, CPEmt1, and CPEmt2. (B) A full-length of ApCPEB4 did not bind o any CPEs and CPEmts (left), whereas a full-length of ApCPEB significantly bound to CPE1, CPE2 and CPE3 but not to CPEmt1 and CPEmt2 (right). (C) A full-length of ApCPEB4 only bound to 1904 sequence but not to 3' UTR of both neuronal actin (left). On the other hand, a full-length of ApCPEB bound to 3' UTR of neuronal actin, but not to 1904 sequence (right).

ApCPEB4 is critical for the initiation of LTF

Previous reports showed that ApCPEB is required for the maintenance of LTF (Si et al., 2003b). I thus examined whether ApCPEB4 plays any specific function during LTF in *Aplysia* by knocking down ApCPEB4 transcripts in *Aplysia* sensory neurons. I generated double-stranded (ds) RNAs against N-terminal sequences of ApCPEB (dsApCPEB) and ApCPEB4 (dsApCPEB4). Each ds RNA was injected into cultured sensory neurons, and the protein level of ApCPEB4 in neurites was measured by immunocytochemistry. Baseline expression as well as 5-HT- mediated translation of ApCPEB4 was significantly blocked in neurons injected with dsApCPEB4, but not in the naïve neurons or neurons injected with dsApCPEB (Naïve: no treatment, 100.0 ± 4.9 %, n = 26 versus 5-HT treatment, 120.9 ± 5.6 %, n = 28; t-test, $p < 0.01$; dsApCPEB: no treatment, 97.1 ± 7.8 %, n = 24 versus 5-HT treatment, 119.8 ± 6.3 %, n = 21; t-test, $p < 0.05$; dsApCPEB4: no treatment, 78.5 ± 5.3 %, n = 19 verse 5-HT treatment, 90.4 ± 5.5 %, n = 20; t-test, $p > 0.05$) (Fig. 14A). These data indicate that dsApCPEB4 specifically blocks both endogenous expression and 5-HT-induced expression of ApCPEB4 in *Aplysia* sensory neurons.

I then examined whether ApCPEB4 is required for LTF. Depletion of ApCPEB during 5-HT exposure to 5x5HT blocks the maintenance, but not the initiation, of the 5-HT- induced LTF (Si et al., 2003b). Interestingly, LTF measured after 24 hrs was significantly impaired in neurons injected with dsApCPEB4, but not in neurons injected with dsApCPEB or dsLuci (dsLuci, 98.7 ± 17.4 %, n = 11; dsApCPEB, 82.3 ± 27.2 %, n = 11; dsApCPEB4, 20.5 ± 18.5 %

EPSP change, n = 12; dsLuci vs. dsApCPEB4, $p < 0.05$ (one-way ANOVA with Tukey-Kramer multiple comparisons)) (Fig. 14B), indicating that ApCPEB4 is involved in the initiation of LTF. This result suggests that the regulation of protein synthesis mediated by ApCPEB4 is critical at the initial stage of LTF formation, whereas ApCPEB is more involved in the long-term maintenance of LTF.


A
Naive
Vehicle
5-HT
dsApCPEB
Vehicle
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dsApCPEB4
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% Fluorescence Intensity
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140
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dsApCPEB4
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% EPSP change
140
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80
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N.S.
dsLuci
dsApCPEB
dsApCPEB4

Figure 14. ApCPEB4, but not ApCPEB is necessary for the initiation of LTF

(in collaboration with Seung-Hee Lee)

(A) The expression and induction of ApCPEB4 following 10 μM 5-HT stimulation was blocked by dsApCPEB4. (Upper) Representative images of neurites of cultured sensory neurons immunostained against ApCPEB4. dsApCPEB showed no effect on the ApCPEB4 expression and induction, while dsApCPEB4 significantly blocked ApCPEB4 expression and induction. Scale bar, 40 μm. (Lower) Bar graphs represent the percent fluorescence intensity of ApCPEB4 in the neurites of naïve, dsApCPEB-injected, dsApCPEB4-injected sensory neurons. 5-HT treatment significantly induced the ApCPEB4 expression which was blocked by injection of dsApCPEB4. (B) LTF at 24 hr was specifically blocked by knock-down of ApCPEB4 (dsApCPEB4). dsApCPEB or dsLuci showed no effect on the 24hr LTF. (Upper) Representative EPSP traces before and 24hr after the 5 pulses of 5-HT treatment at the sensory-to-motor synapses. (Lower) Bar graph represents the means ± SEM of the percent change in EPSP amplitude. *, $p<0.05$; **, $p<0.01$; N.S., not significant.

Overexpression of ApCPEB4 reduces the threshold of LTF induction

I further examined a specific role of induced ApCPEB4 by overexpressing it directly in sensory neurons of sensory-motor cocultures (Fig. 15A). Interestingly, 1X 5-HT treatment (10 μM, 5 min), which normally induces short-term facilitation (STF), induced LTF by overexpression of ApCPEB4, but not EGFP in sensory neurons (EGFP, 21.1 ± 20.4 %, n = 11; ApCPEB4 + EGFP, 111.0 ± 27.5 % EPSP change, n = 13; t-test, $p < 0.05$) (Fig. 15B). These results suggest that the overexpression (artificial induction) of ApCPEB4 reduced the threshold of LTF induction and thus induced LTF with single pulse of 5-HT stimulus, further supporting the idea that the translational induction of ApCPEB4 is critical for the formation of LTF in *Aplysia*.

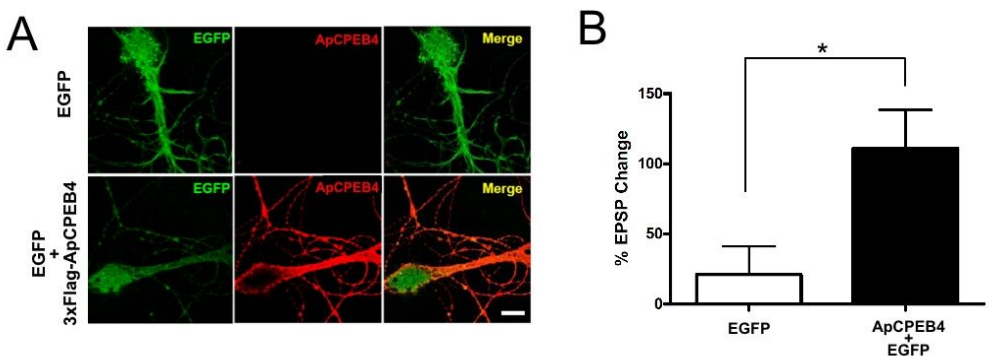


Figure 15. Overexpression of ApCPEB4 is sufficient to induce LTF with one pulse of 5-HT

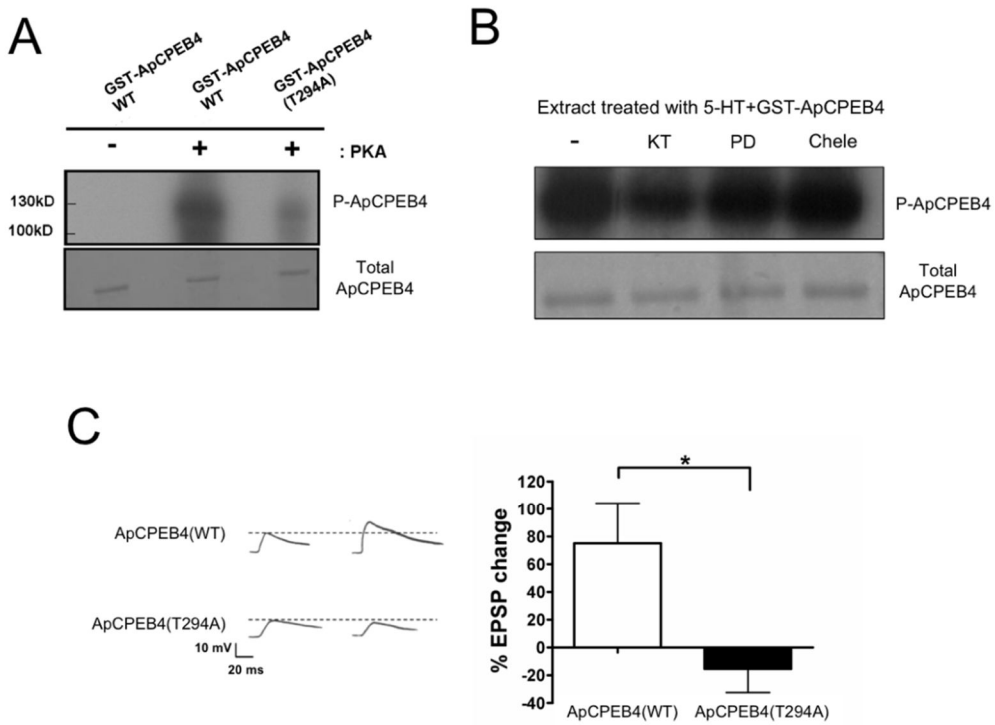
(A) Overexpressed 3xFlag-ApCPEB4 in cultured sensory neurons was detected by anti-Flag antibody. As a control, EGFP-expressing sensory neurons were used. Scale bar, 20 μm. (B) The overexpression of ApCPEB4 induced LTF by 1X 5-HT treatment. As a control, EGFP was expressed. Bar graph represents the means ± SEM of the percent change in EPSP amplitude. *, $p<0.05$.

Phosphorylated ApCPEB4 by PKA is critical for LTF induction

Previous report showed that ApCPEB is not phosphorylated by PKA (Si et al., 2003b). On the other hand, ApCPEB4 possesses one conserved putative PKA phosphorylation site on the 294th threonine residue (Fig. 16A). Thus, I hypothesized that the function of ApCPEB4 might be regulated by PKA-mediated phosphorylation. We first performed an *in vitro* kinase assay. Purified GST-ApCPEB4 fusion proteins were phosphorylated by the catalytic subunit of PKA *in vitro* (Fig. 16A). The phosphorylation was reduced in the non-phosphorylatable mutant form of ApCPEB4 (ApCPEB4 T294A), in which 294th threonine was replaced by alanine (Fig. 16A). These results indicate that the 294th threonine of ApCPEB4 is a potential PKA phosphorylation site. In addition, we found that ApCPEB4 was phosphorylated by *Aplysia* neuronal cell lysate in a PKA-dependent manner (Fig. 16B), indicating that ApCPEB4 is a genuine substrate of endogenous PKA in *Aplysia* neurons.

Next, I wanted to test whether this phosphorylation of ApCPEB4 by PKA is critical for the initiation of LTF. To answer the question, I overexpressed the ApCPEB4(T294A) in Aplysia sensory neurons cocultured with motor neurons because the mutant form ApCPEB4(T294A) should act as a dominant negative mutant if the phosphorylation of ApCPEB4 on the 294th threonine is critical. I found that LTF was completely blocked in the synapse overexpressed with ApCPEB4(T294A) in sensory neurons, whereas expression of ApCPEB4-WT control did not affect the LTF (ApCPEB4(WT), 75.0 ± 29.4 %, n = 10 versus

ApCPEB4(T294A), -15.4 ± 17.0 % EPSP change, n = 5, t-test, $p < 0.05$) (Fig. 16C). Taken together, phosphorylation of ApCPEB4 by PKA is required for the induction of LTF in *Aplysia*.


A
GST-ApCPEB4 WT
GST-ApCPEB4 WT
GST-ApCPEB4 (T294A)
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+
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: PKA
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P-ApCPEB4
Total ApCPEB4
B
Extract treated with 5-HT+GST-ApCPEB4
-
KT
PD
Chele
P-ApCPEB4
Total ApCPEB4
C
ApCPEB4(WT)
ApCPEB4(T294A)
10 mV
20 ms
% EPSP change
120
100
80
60
40
20
0
-20
-40
*
ApCPEB4(WT)
ApCPEB4(T294A)

Figure 16. Phosphorylation of ApCPEB4 is required for both the LTF formation

(A) *In vitro* phosphorylation assay showed that purified ApCPEB4 was directly phosphorylated by PKA on its 294th threonine residue.

(B) Phosphorylation of purified ApCPEB4 was examined using *Aplysia* pleural ganglia extracts as an endogenous mixture of kinases. Concurrent treatment of 40 μM KT5720, a PKA inhibitor, significantly reduced the amount of phosphorylation on ApCPEB4. Neither 20 μM PD98059 (PD), a MEK inhibitor, nor 10 μM chelerythrine (Chele), a PKC inhibitor, affected the phosphorylation of ApCPEB4.

(C) Phosphorylation of ApCPEB4 on its 294th threonine residue was required for the LTF formation. (C, left) Representative traces of EPSP measured at the sensory-to-motor synapses before and 24 hr after the 5 pulses of 5-HT. (C, right) Bar graph represents the mean percentage change ± SEM in EPSP amplitude. Overexpression of ApCPEB4(T294A), non- phosphorylatable mutant of ApCPEB4, significantly blocked LTF. *, $p < 0.05$.

DISCUSSION

In this study, we cloned a novel protein ApCPEB4, which is related to ApCPEB. The translational increase of ApCPEB4 was critical for the formation of LTF, and overexpression of ApCPEB4 reduced the threshold for the LTF. In addition, phosphorylation of ApCPEB4 by PKA was required for the LTF formation. Combined, our results suggest that ApCPEB4 and ApCPEB work cooperatively in different stages of LTF. The former works for initiating the long-term facilitation and latter works for maintaining it.

ApCPEB4 is essential for the initiation of LTF : different ApCPEBs regulate distinct target mRNAs during LTF

Our data revealed an involvement of ApCPEB4 in the initiation of LTF, and that the overexpression of ApCPEB4 reduces the threshold of LTF induction. This is in contrast to another *Aplysia* CPEB, which can regulate the maintenance of LTF at 72 hr. Thus the two ApCPEBs play distinct roles in 5-HT-induced LTF.

How do these two ApCPEBs regulate LTF formation and maintenance differentially? One of the plausible explanations is that these two ApCPEBs have different RNA binding specificity. We found that ApCPEB but not ApCPEB4 binds to CPE sequence as well as 3' UTR of actin in CPE-dependent manner (Fig.

13). By contrast, ApCPEB4 bound to a different U-rich sequence, the 1904 sequence, which is a synthetic binding sequence for mammalian CPEB3 and CPEB4, but not a canonical CPE (Fig. 13) (Huang et al., 2006a). In fact, mammalian CPEB1 and other mammalian CPEB2-4 also have different target mRNAs to regulate translation for different stages of synaptic plasticity via CPE site-dependent and-independent manners, respectively (Huang et al., 2006a).

These observations, suggest that activated ApCPEB and ApCPEB4 may regulate protein synthesis of two distinct groups of mRNAs, one group of mRNAs containing CPE sites for the maintenance of LTF and another group of mRNAs containing CPE-independent sites for the initiation of LTF. It would be interesting to further discriminate target mRNAs used for distinct phases of LTF that are translated by ApCPEB and ApCPEB4, respectively.

PKA-dependent activation of ApCPEB4

In *Xenopus* oocytes, CPEB1 is phosphorylated by the kinase Aurora A (Eg2) at a canonical LD(S/T)R site (Mendez et al., 2000a; 2000b), and the phosphorylation of CPEB1 binds to cleavage and polyadenylation specificity factor (CPSF) to induce release of PARN from the ribonucleoprotein (RNP) complex, thereby enabling Germ-line-development factor 2 (Gld2) to elongate poly(A) tailing by default (Mendez et al., 2000b). On the other hand, ApCPEB has been found not to be phosphorylated but to be increased in the amount of protein expression to enhance the affinity to CPSF (Si et al., 2003b). Interestingly,

ApCPEB4 is regulated differentially from ApCPEB. ApCPEB4 is directly phosphorylated by PKA on its canonical LD(S/T)R site.

In *Aplysia*, PKA is critical for both synapse-specific and cell-wide facilitation induced by 5-HT signaling. PKA phosphorylates many components required for LTF formation in *Aplysia* such as cAMP response element-binding protein (CREB), synapsin, *Aplysia* Activating Factor (ApAF), and Cell Adhesion Molecule-Associated Protein (CAMAP)(Kaang et al., 1993; Bartsch et al., 2000; Angers et al., 2002; Lee et al., 2006; 2007). Although I do not provide direct evidence, this study provide further insight into the mechanism of how the long-lasting forms of synaptic plasticity can be initiated via PKA-mediated phosphorylation and local translation of ApCPEB4. ApCPEB4 might connect PKA signaling to the local protein synthesis, which is required for the induction of more sustained synaptic activation, by means of the enhanced expression of target mRNAs of ApCPEB4 to support 5-HT-induced LTF.

Possible roles of ApCPEB4 in synapse-specific LTF

As shown in Figure 2, ApCPEB4 protein can be localized in neurites. In addition, we previously reported that ApCPEB4-EGFP could form RNA granules within the neurites in *Aplysia* sensory neurons (Chae et al., 2010). Combined, ApCPEB4 can be localized in neurites and involved in local protein synthesis.

During synapse-specific LTF, local protein synthesis is required for two distinct phases of LTF: initiation and maintenance (Martin et al., 1997b; Casadio et

al., 1999). For the maintenance of synapse-specific LTF, a rapamycin-sensitive local protein synthesis is required (Martin et al., 1997a; Casadio et al., 1999). One essential molecule which is locally synthesized in a rapamycin-sensitive manner is ApCPEB. ApCPEB regulates local translation of many specific mRNAs containing CPE sites including actin mRNA to sustain the synaptic facilitation for periods up to 72 hrs by supporting persistent structural and functional changes of the synapses (Miniaci et al., 2008b). However, for the initiation of LTF, a second, rapamycin-insensitive, emetine-sensitive component of local protein synthesis is required in synapse-specific LTF (Casadio et al., 1999). This study showed that local induction of ApCPEB4 by 5-HT treatment is rapamycin-insensitive and emetine-sensitive. In addition, I also found that one pulse of 5-HT produced LTF in ApCPEB4-overexpressing sensory neurons. It is therefore possible that overexpression of ApCPEB4 combined with one pulse of 5-HT may be sufficient to produce the retrograde signal required for LTF induction. Overall, ApCPEB4 may be a key regulator required for generating the retrograde signal in initial local protein synthesis during synapse-specific LTF. Although it is still possible that ApCPEB4 may be involved in the rapamycin-insensitive, emetine-sensitive intermediate-term facilitation (ITF) (Yanow et al., 1998; Jin et al., 2011), it would be interesting to further dissect this possibility in a synapse-specific form of LTF.

CHAPTER IV

CONCLUSION

CONCLUSION

In this study, I investigated mechanisms of memory consolidation and reconsolidation at the molecular and cellular level using *Aplysia* sensory-to-motor neuron coculture system.

In Chapter II, I focused on the cellular mechanisms of memory reconsolidation using *Aplysia* sensory-to-motor coculture system. In this study, I firstly report that that synaptic destabilization and restabilization occur at the same synapse where it stored memory before that retrieval. Using emetine, which is protein synthesis inhibitor I first confirmed that synapse become labile after receiving memory reactivating or retraining signal in the *Aplysia* culture system as it is reported previously in mouse (Lee et al., 2008a; Kim et al., 2010). This decrease in synaptic strength was retrieval specific because it did not decrease only by emetine without retrieval signal. As it is reported in mouse hippocampus (Lee et al., 2008a), treatment of βlac together with emetine during retrieval signal, blocked synapse becoming labile. By using *Aplysia* monosynaptic coculture system, I found that synaptic destabilization and restabilization occur in the same synapse.

In Chapter III, I focused on the function of a novel CPEB isoform in *Aplysia*, ApCPEB4, in long-term facilitation(LTF) which is a cellular analog of memory consolidation. ApCPEB4 was name based on the RRM sequence which showed higher similarity to CPEB2-4 family other than that of ApCPEB. Expression of ApCPEB4 was highly enriched in CNS and it was increased by

application of 5-HT which induce LTF. Next I also found that translation of ApCPEB4 was locally regulated and it was transcription-independent, PKA-dependent, emetine-dependent and rapamycin-independent. Interestingly, ApCPEB4 and ApCPEB have distinct RNA binding selectivity. ApCPEB4 was bound to non-CPEB U-rich SELEX sequence, which is a target of mammalian CPEB2-4 (Huang et al., 2006a) but it did not bound to canonical CPE sequence in Actin 3'-UTR which is a target of ApCPEB (Liu and Schwartz, 2003a; Si et al., 2003a). Using double stranded RNA, expression level of ApCPEB4 was decreased and this impaired formation of LTF. On the other hand, Overexpression of of ApCPEB4 in sensory neuron reduced threshold for the LTF induction. And I also found that phosphorylation of ApCPEB4 by PKA was critical for LTF induction. Taken together, ApCPEB4 plays a key role in the initiation of LTF in *Aplysia*.

Further studies will address pile of questions about memory consolidation and reconsolidation. Profiling of proteins synthesized during consolidation and reconsolidation will give more information to understand this process. In addition, finding the specific role of ApCPEB4 by verifying the target mRNA will give more clear mechanisms of ApCPEB4 in memory consolidation.

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국문초록

단기기억이 장기기억으로 저장되기 위해서는 단백질 합성을 필요로 하는 경화과정이 필요하다. 기억 재경화 가설은 경화된 장기기억이 회상을 통해 떠올려지며 그 후 재경화 과정을 거쳐야만 다시 안정화 될 수 있다고 설명한다. 하지만 회상에 의해 기억이 인출되는 과정 중 시냅스에서 일어나는 단백질 분해와 재합성 과정이 동일한 시냅스에서 일어나는지에 대해서는 아직 확실하게 알려져 있지 않았다. 이러한 현상을 자세히 밝히기 위해 비교적 단순한 신경계로 구성된 바다달팽이의 민감화기억에 초점을 맞추기로 하였다. 이러한 민감화 기억은 군소 단일신경세포 배양법을 이용해 장기기억이 장기 시냅스 촉진으로 세포수준에서 재현될 수 있다. 이번 연구를 통해 기억의 회상에 의해 일어나는 시냅스 단백질의 분해와 재합성이 장기 시냅스 촉진이 일어나는 동일한 시냅스에서 일어나는 것을 확인하였다.

그 외에도, 군소의 시냅스 특이적인 장기 시냅스 촉진 과정의 형성과 유지에 필요한 국소 단백질 합성 과정이 각각 다른 신호전달 경로로 일어나는 것에도 관심을 가졌다. 기존 연구들을 통해 ApCPEB 이라는 단백질은 rapamycin에 의해 억제되는 단백질 합성 경로를 통해 형성되며 이것의 발현이 장기 시냅스 촉진과정의 유지에 필요한 것으로 알려져 있다. 그러나 emetine에 의해 억제되는 단백질 합성 경로를 통한 장기 시냅스 촉진의 형성에 필요한 국소 단백질 합성 과정은 아직 알려

져 있지 않았다. 이 논문에서는 기존에 알려져 있지 않던 새로운 Aplysia CPEB를 클로닝 하였고, ApCPEB4-like protein 이라 명명하였다. 이 단백질은 5-HT에 의해 증가하였고, 프리온 도메인이 없었다. 또한 emetine에 의해 합성이 억제 되지만 rapamycin에 의해서는 합성이 억제되지 않는 것을 확인할 수 있었다. ApCPEB4는 기존에 알려져 있던 ApCPEB와 다른 target mRNA에 붙는 것으로 확인 되었고, 이 단백질의 발현을 저해하였을 때 장기 시냅스 촉진의 형성이 억제되었다. 또한, 과발현 시에는 장기 시냅스 촉진이 형성되기 위한 역치값이 감소한 것을 확인하였다. 이러한 결과들을 통해 ApCPEB은 장기시냅스 촉진의 유지를 위해 필요한 반면, 프리온 도메인이 없는 ApCPEB4는 장기 시냅스 촉진의 형성에 중요한 것을 알 수 있었다.

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주요어 :

CPEB, 장기시냅스촉진, 국소단백질합성, 역방향신호전달, 흥분성시냅스후전압

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